Vol. 48, 1972

Acta Haematologica

International Journal of Haematology
Journal International d Hématologie
Internationale Zeitschrift für Hämatologie

Official Organ of the European Division of the International Society of Haematology



S. Karger · Basel · München · Paris · London · New York · Sydney

Index

S Karger Basel München Paris London New York Sydney
Arn ld Böckl n S rasse 25 CH–4011 Basel (Sw tzerland)

Acta Haemat 48 1 11 (1972)

Hereditary Elliptocytosis

Haematological and Metabolic Findings[1]

G TORLONTANO, L. FONTANA, A DE LAURENZI, G PAPA and M PROIETTI

Haematology Unit, Medical Clinic II Medical Pathology I and Department of Haematology, University of Rome Rome

Abstract The examination of the erythrocyte biochemistry in 15 cases of hereditary elliptocytosis has shown a constant and significant increase of GSH both before and after incubation with APH and an increase of G6PDH, NAPDH GR, HK, PFK, ENOL, and LDH activities A slight decrease in ALD and ATP ase was also observed. The raised values of many enzymatic activities may be a consequence of hyperhaemolysis and thus the presence of a young erythrocyte population containing an increased concentration of enzymes. The normal but low values of ATP ase and the ALD deficiency may be the result of structural alterations of the erythrocyte membrane

Key Words
Elliptocytosis
Elliptocyte biochemistry
Erythrocyte enzymes

Hereditary elliptocytosis is an ubiquitous, inherited autosomal erythropathy It is characterized by the presence of generally more than 20–25%, and often more than 90%, of oval and elliptical erythrocytes in stained blood samples [30, 31], moreover, it is generally characterized by normal values of MCV, MCH and MCHC, and often by increased osmotic fragility of RBC [18]

The instances of variability of this disorder suggest that several genes of different activity are responsible for the erythropathy· at least one of these genes is certainly linked to the Rh locus [18] It has been found, however, that even a single elliptocythaemic gene through its varied possibilities of expression as a dominant gene can determine a wide range of conditions, from latent to haemolytic and anaemic clinical forms in the same family [13 14 28 42]

[1] Paper presented to the 1st Meeting of the European Division of the International Society of Haematology, Milano 10–12 September 1971

Erythrokinetic studies have clarified several pathogenetic features of the disease, such as the generally fundamental role of the spleen in erythrocyte sequestration and destruction [11, 32] and particularly the presence, in some anaemic subjects, of ineffective erythropoiesis [10]. Moreover, biometric studies of the spleen associated with erythrokinetic studies have revealed hypersplenism in some elliptocytic anaemic subjects [48].

The nature of the disorder remains uncertain. DE GRUCHY *et al.* [21] have demonstrated an increased rate of fall in ATP and 2,3-diphosphoglycerate concentrations and a faster rise in inorganic phosphate concentration, in elliptocytes, by the incubation of blood at 37 °C. Moreover, PETERS *et al.* [41] have shown that active sodium efflux is significantly greater in elliptocytic erythrocyte membranes. This, according to the authors, suggests a membrane abnormality responsible for the increased sodium transport. A primitive membrane defect has also been postulated by MURPHY [37] on the basis of the abnormal location of cholesterol on the erythrocyte membrane, and by RIEBUCK and VAN SLYCK [46] on the basis of the particular disposition of the erythrocyte haemoglobin, revealed by electron microscopy.

Studies on the enzymatic pattern of erythrocytes, carried out on elliptocytemic subjects, are still generally limited to a few enzymes and are mostly carried out on single cases [16, 17, 20, 38–40, 43, 44]. PUXEDDU *et al.* [44] have found normal values of erythrocyte enzymes in 2 elliptocythaemic subjects, without signs of hyperhaemolysis, and elevated enzymatic values in a subject with haemolytic elliptocytosis. Normal enzymatic behaviour has been found in one [16] and 3 [38] subjects, with a combination of hereditary elliptocytosis and heterozygous β-thalassaemia. OZER and MILLS [39], PRYOR and PITNEY [43], and PEARSON [40] have observed an associated deficiency of G6PDH in some elliptocythaemic subjects; further, subnormal GSH values have been reported in one case each by OZER and MILLS [39] and DAVOLI *et al.* [20]. Some of these authors [20, 39] have suggested that the presence of hyperhaemolysis in cases of hereditary elliptocytosis may be due to occasional enzymatic deficiencies of the described type or to other associated metabolic disturbances, varying from case to case.

The present study was carried out in order to further clarify the pathogenesis of hereditary elliptocytosis. Evaluations were made of reduced glutathione and its stability, and 13 erythrocyte enzymes were determined, together with the haematologic studies in 15 subjects affected by the condition.

Material and Methods

The 15 elliptocythaemic subjects examined (9 males and 6 females) were members of 6 different families with confirmed elliptocythaemic tara (table I). Two of

these subjects (cases IV-1 and V-1) were anaemic A third case (II 1) had suffered from hyperhaemolysis with anaemia and, following successful splenectomy, at the time of this study, haemoglobin and red blood cell values were normal

The haematological studies were carried out according to standard techniques [19] The electrophoretic haemoglobin study was carried out by starch block electrophoresis, according to the technique of GERALD and DIAMOND [24]

The morphological erythrocyte study was conducted with smears stained by May Grunwald-Giemsa solution, and the degree of excentricity was calculated according to GÜNTHER's formula

$$\varepsilon = 1 - \sqrt{\left(\frac{b}{a}\right)^2},$$

where 'a' is the longer, and 'b' the shorter axis [25] In this way, the erythrocytes could be divided into 4 classes class I (round), $\varepsilon < 0.47$, class II (nearly round), $\varepsilon = 0.47$–0.62, class III (broad elliptical), $\varepsilon = 0.62$–0.74, class IV (narrow elliptical), $\varepsilon > 0.74$ In normal subjects there are no more than 10 to 15% of erythrocytes of classes III and IV

In cases I 1, II 1, II 2, II 3, and V-1, the erythrocyte half survival time was studied, the erythrocytes were labeled with ^{51}Cr and re injected according to the method used in this laboratory, by which the normal range of $T^{1}/_{2}{}^{51}Cr$ in healthy subjects is 28 to 35 days [13]

In all cases, the following substances and enzymes were tested

(1) reduced glutathione (GSH) and its stability after incubation with acetylphenylhydrazine (APH), according to BEUTLER *et al* [4],

(2) glucose-6-phosphate dehydrogenase (G6PDH), according to the method of KORNBERG and HORECKER [29],

(3) NADH glutathione reductase (NADH GR) and NADPH glutathione reductase (NADPH GR) by RACKER's method [45],

(4) enzymes of the Embden Meyerhoff Parnas glycolytic cycle hexokinase (HK), according to CRANE and SOLS [15], phosphofructokinase (PFK), according to LING *et al* [33] aldolase (ALD), according to BRUNS [6], triosophosphate isomerase (TPI), according to BEISENHERZ [2], phosphoglyceraldehyde phosphate dehydrogenase (GAPDH), according to BEISENHERZ *et al* [3], enolase (ENOL), according to BÜCHER [7], pyruvate kinase (PK), according to BÜCHER and PFLEIDERER [8], and lactate dehydrogenase (LDH), according to WROBLESWSKI and LA DUE [52],

of the techniques [22, 34]

The results obtained were elaborated statistically The differences of the means were tested for significance by Student's t test

Results

In 11 out of the 14 non-splenectomized subjects, a generally slight enlargement of the spleen was observed clinically (table I) The haematolog-

Table I Haematological data in 15 subjects with hereditary elliptocytosis

Case No	Sex	Hb, g/100 ml	RBC, ×10⁶	Reticulocytes, %	MCV, μm^3	MCH, pg	MCHC, %	Osmotic fragility	Elliptocytes, classes III and IV [25], %	Hb A_2, %	Total bilirubin, mg/100 ml	T½ ^{51}Cr	Splenomegaly[2]
I-1	M	12.5	4.3	3.7	90	29	32	increased	87	1.95	3.70	16.0	++
I-2	M	12.5	4.4	2.4	90	28	32	increased	89	2.30	0.70	–	+
I-3	M	13.0	4.5	2.1	84	29	34	increased	94	1.90	0.64	–	+
II-1	M	16.0	5.8	1.1	83	27	33	increased	84	2.22	0.55	27.5[1]	splenectomiz.
II-2	F	13.0	4.7	2.5	87	28	32	increased	92	2.15	0.75	18.5	++
II-3	F	12.0	4.5	3.1	87	27	31	increased	98	2.18	0.73	17.0	+
II-4	F	12.5	4.8	2.5	87	26	30	increased	80	2.20	0.70	–	+
III-1	F	13.0	4.5	2.4	93	29	31	normal	40	1.40	0.60	–	+
III-2	M	16.0	5.3	2.5	90	30	33	normal	24	2.38	0.70	–	+
III-3	M	15.5	4.9	2.2	92	32	34	normal	30	2.24	0.60	–	+
III-4	F	14.0	4.8	1.0	92	29	32	normal	32	2.93	0.62	–	–
III-5	M	15.0	5.3	2.0	87	28	33	normal	20	1.80	0.75	–	–
IV-1	M	10.5	4.0	2.3	90	26	29	normal	35	2.10	2.50	–	++
V-1	F	9.1	3.0	3.1	100	31	31	normal	53	1.90	2.10	10.5	+++
VI-1	M	14.9	4.8	2.4	90	31	35	normal	42	1.68	1.10	–	+

[1] After splenectomy (before splenectomy T½ ^{51}Cr 9.5 days)

[2] + Spleen hardly palpable under the costal arc, ++ spleen palpable at 2–3 cm under the costal arc, +++ spleen palpable beyond 3 cm under the costa arc.

ical study showed, first of all, an anaemic state in cases IV-1 and V-1, while subject II-1, formerly haemolytic and anaemic, had been splenectomized and presented normal erythrocyte and haemoglobin values

Elliptic deformation of the erythrocytes ranged from a minimum of 20% (case III-5) to a maximum of 98% (case II-3) (table I) The MCV, MCH and MCHC were usually found to be normal Slightly subnormal MCH and MCHC values were observed only in cases II-4 and IV-1

The electrophoretic study of haemoglobin and the percentage evaluation of HbA_2 presented normal values in all cases

The osmotic fragility of erythrocytes was found to be increased in 7 subjects (cases I-1–II-4), while normal in the others The percentage of reticulocytes was found to be higher than the normal value of 2 0% in nearly all cases An increase in bilirubin was observed in cases I-1, IV-1, and V-1

The study of erythrocyte half survival time, carried out in 5 subjects, produced clearly subnormal values in cases I-1, II-2, III-3, and V-1, and slightly reduced values in case II-1, the splenectomized subject The erythrocyte half survival time values in this patient, however, corresponded to $T^1/_2$ of 9 5 days before splenectomy [49]

With regard to erythrocyte biochemistry, significantly higher values than normal were found for GSH, G6PDH, NADPH-GR, HK, FPK, ENOL, and LDH Raised GSH values were found both before and after incubation with APH in all cases, the mean values were, respectively, 108 133±15 027 and 104±13 953 mg/100 ml RBC (normal mean values 74 20±10 67 and 70 50±10 50) (table II) The statistical differences were highly significant, with t=9 3582 and p<0 0005 for GSH values before incubation with APH, and t=9 6101 and p<0 0005 after incubation (table II)

G6PDH activity was increased in 13 out of the 15 cases studied, with a mean value of 1,478 266±393 12 units (normal mean value 1,074±279, t=4 2630 and p<0 0005) (table II) HK activity was increased in 13 cases, with a mean value of 139 733±48 263 and p<0 001 (table II) PFK activity was increased in all cases, with a mean value of 1,880+666 units (normal mean value 1,328±223 units, t = 3898 and p<0 001) (table II)

Finally, ENOL and LDH were increased in 13 cases, the first enzyme with a mean value of 3,986 333±1,140 70 units (normal value 2,719±624 units), t=4 0800 and p<0 0005, the second enzyme, with a mean value of 36,012±6 632 31 units (normal value 31,039±4 611,

Table II Metabolic findings

	Normal subjects, 40 cases	Patients, 15 cases	t	p level of significance
	Mean ± SD	Mean ± SD		
GSH				
before incubation	74.20 ± 10.67	108.133 ± 15.027	9.3582	<0.0005
after incubation	70.50 ± 10.50	104.000 ± 13.953	9.6101	<0.0005
G6PDH	1,074.00 ± 279	1,478.266 ± 393.12	4.2630	<0.0005
NADH-GR	301.00 ± 114	346.466 ± 85.48	1.4007	>0.05
NADPH-GR	957.00 ± 122	1,166.533 ± 167.93	5.1017	<0.0005
HK	91.00 ± 25	139.733 ± 48.263	3.7276	<0.001
PFK	1,328.00 ± 233	1,880.666 ± 530.24	3.893	<0.001
ALD	493.00 ± 107	406.800 ± 86.953	2.7888	<0.005
TPI	64,532.00 ± 9,619	60,958.266 ± 14,890.1	0.8644	>0.05
GAPDH	6,544.00 ± 1,122	6,420.066 ± 1,568.67	0.3260	>0.05
ENOL	2,719.00 ± 624	3,986.333 ± 1,140.70	4.0900	<0.0005
PK	2,795.00 ± 422	3,342.733 ± 1,324.29	1.5701	>0.05
LDH	31,039.00 ± 4,611	36,012.012 ± 6,632.31	3.1456	<0.0005
ATP-ase	12.00 ± 1.73	9.068 ± 0.55045	12.6907	<0.0001
Acid phosphatase	71.66 ± 12.48	76.836 ± 8.3187	1.48301	>0.05

t=3 1456 and p<0 0005) (table II) NADH-GR, PK and acid phosphatase revealed a barely significant increase (table II) Similarly, decreases in the mean TPI and GAPDH activity were scarcely significant (table II)

A statistically significant decrease in ALD and ATP-ase values was found ALD activity was slightly decreased, with a mean value of 406 8±96 953 units (normal mean value 493±107), t=2 7888 and p<0 005, ATP ase activity was found to be barely normal in all cases, with a mean value of 9 068±0.55045 μM (normal mean value 12 98±1 73 μM, t=12 6907 and p<0 0001) (table II)

Discussion and Conclusions

The haematological study revealed a significant occurrence of anaemic subjects and, in nearly all the subjects examined, hyper-reticulocytosis and an enlarged spleen, which may be related to hyperhaemolysis This condition was further confirmed in all subjects in which the study of erythrocyte survival, using ^{51}Cr, could be carried out. This finding confirms the results of earlier reports [11, 13, 14, 23, 28, 49] of an elevated frequency of symptoms observed in subjects affected by hereditary elliptocytosis

The studies on erythrocyte biochemistry showed, in addition to statistically insignificant variations of the enzymatic activity of NADH-GR, TPI, PK, GAPDH, and acid phosphatase, a constant and significant increase of GSH both before and after incubation with APH, and an increase in G6PDH, NADPH, HK, PFK, ENOL, and LDH activity Further, a slight but significant decrease in ALD and ATP-ase was observed

The results of this study show no striking biochemical defects which might characterize hereditary elliptocytosis Moreover, the total absence of occasional striking enzymatic defects excludes the possibility of a causal influence of occasional associated enzymatic defects in the genesis of hyperhaemolysis in the cases studied This possibility had been suggested by other authors [20, 39]

Similarly, on the basis of previous experiments, it seems improbable that the genes for *HbS* [12, 51], *HbC* [36], for thalassaemia [1, 16] and the hereditary persistence of fetal haemoglobin [47] interact with the gene for elliptocytosis and worsen its clinical and haematological picture

The increase in many enzymatic activities, observed in nearly all cases studied, can be considered a consequence of hyperhaemolysis and of the

presence, therefore, of a young, enzyme-rich erythrocyte population in the peripheral blood [9, 35]

The barely normal ATP-ase activity in all cases is, thus far, of uncertain significance and must be studied in more detail It could be an expression of a structural alteration of the erythrocyte membrane [5], a primitive defect of which, as mentioned before, has been suggested by MURPHY [37], PETERS *et al* [41] and REBUCK and VAN SLYCK [46] The slight but significant defect of ALD activity also deserves further attention This could be an artifactual result of the abnormal shape of erythrocytes, similar to the finding of HANES *et al* [26] with regard to hereditary spherocytosis

In conclusion, these data further confirm the elevated frequency of hyperhaemolysis in hereditary elliptocytosis The illness, however, seems to be attributable neither to a primitive enzymatic defect nor to an occasional coincidence of enzymatic alterations It is possible that, in individual cases, the varying degree of expressivity of elliptocytic genes may condition the intensity of the pathologic manifestations, due to a primitive defect of the erythrocyte membrane

Acknowledgement We are indebted to Drs A ZULIANI and G TROI of the University of Rome for the statistical analysis of the data.

References

1 AKSOY M Combination of hereditary elliptocytosis with heterozygous beta thalassemia. Study of a Turkish family Acta haemat., Basel *30* 215–220 (1963)

2 BEISENHERZ, G Triosephosphate isomerase from calf muscle in COLOWICK and KAPLAN Methods in Enzymology, vol 1, pp 387–391 (Academic Press, New York 1955)

3 BEISENHERZ, G BOLTZE, H , BUCHER, T , CZOK, R GARBADE, K H MEYER ARENDT, E. und PFLEIDERER G Diphosphafructose Aldolase, Phosphoglycer aldehyd Dehydrogenase Milchsäure-Dehydrogenase Glicerophosphat Dehydrogenase und Pyruvat Kinase aus Kaninchen-Muskulatur in einem Arbeitsgang Z. Naturforsch. *8* 555–577 (1953)

4 BEUTLER, E., DURON O., and KELLY, B M Improved method for the determination of blood glutathione J Lab clin Med *61* 882 888 (1963)

5 BRUNETTI, P PUXEDDU A SANTEUSANIO F DE CINCES, P , SIRACUSA A , AR[illegible] D e MIGLIORINI, E. L anemia emolitica da d fetto di piruvico-chinase (PK) S ud o patogeneti o Ric cl n lab *2* 3-23 (1969)

6 BRUNS F. Über d e Aldolase der Erythrocyten. B ochem Z. *325* 429–438 (1954)

7 BUCHER T Enolase from brewer s yeast in COLOWICK and KAPLAN Methods in enzymology, vol 1, pp 427–435 (Academic Press, New York 1955)

8 BUCHER, T and PFLEIDERER, G Pyruvate kinase from muscle in COLOWICK and KAPLAN Methods in enzymology, vol. 1, pp 435–440 (Academic Press, New York 1955)

9 BUTENARDT, O The activity of glutathionperoxydase, glucose-6-phosphate-dehydrogenase and glutathionreductase in spherocytes. Acta haemat., Basel *43* 348–352 (1970)

10 CAMPANACCI L., TORLONTANO, G, TONIETTI, G., and CONTI C. Erythrokinetics in hereditary elliptocytosis. Proc. 7th Congr Europ Soc. Haemat., London 1959 Part 2, pp 1099–1103 (1960)

11 CASSANO C., CONTI, C. e TORLONTANO, G Fattori di 'scompenso' nella ipere molisi costituzionale Recenti Progr Med *30* 101–131 (1961)

12 CONTI, C., MUZZOLINI, M e TORLONTANO G La malattia ellittocitemica. Nota II Associazione ellittodrepanocitica. Rass Fisiopat clin. ter *21* 665–687 (1959)

13 CONTI, C. e TORLONTANO G La ellittocitosi constituzionale studio clinico e fi siopatologico 19th Congr Soc Ital. Haemat., Pavia 1963 (Viscontea, Pavia 1963)

14 CONTI, C. TORLONTANO, G e MUZZOLINI, M Dinamica della emolisi ed aspetti ematologici nella malattia ellittocitemica Policlinico, Sez. prat. *69* 1477–1497 (1962)

15 CRANE, R. K. and SOLS A Animal tissue hexokinase, in COLOWICK and KAPLAN Methods in enzymology, vol 1, pp 277–286 (Academic Press, New York 1955)

16 CUTILLO, S, COLETTA, A., LUPI, L. e SCALA, M Associazione dell ellittocitosi al tratto talassemico Pediatria, Napoli *70* 269–280 (1962)

17 CUTTING H. O, MCHUGH, W, CONRAD, F G., and MARLOW, A. A. Autosomal dominant haemolytic anemia characterized by ovalocytosis a family study of seven involved members. Amer J Med. *39* 21–34 (1965)

18 DACIE, J V The haemolytic anaemias Congenital and acquired I The congen ital anaemias 2nd ed, pp 151–170 (Grune & Stratton New York 1960)

19 DACIE, J V and LEWIS, S M Practical haematology, 4th ed. (Churchill, London 1968)

20 DAVOLI, P G TRALDI, A. e BALDUINI, C. Livello di attività di alcuni enzimi del metabolismo energetico e tasso di glutatione ridotto negli eritrociti di un pa ziente affetto da ellittocitosi ereditaria e nei suoi familiari Boll Soc ital Biol sper *44* 719–721 (1968)

21 DE GRUCHY, G C., LODER, R. B., and HENNESSY, I V Haemolysis and glycolytic metabolism in hereditary elliptocytosis Brit. J Haemat. *8* 168–179 (1962)

22 FONTANA, L., PAPA, G, DE LAURENZI, A. e LIOTTA, S Il biochimismo eritrocitario nella cirrosi epatica di Morgagni Laennec. Med clin sper *17* 376–396 (1967)

23 GEERDINK, R. A., HELLEMAN, P W., and VERLOP M C. Hereditary elliptocytosis and hyperemolysis A comparative study of 6 families with 145 patients Acta med scand *179* 715–728 (1966)

24 GERALD P S and DIAMOND L. K. The diagnosis of thalassemia trait by starch block electrophoresis of the hemoglobin Blood *13* 61–69 (1958)

25 GÜNTHER H I Formprobleme an menschlichen Erythrocyten Folia haemat., Lpz. 35 383–417 (1925)
26 HANIS T E, PATERSON J, and VAN EYS, J Red cell aldolase and other enzyme activities in hereditary spherocytosis J Lab clin Med 75 654–658 (1970)
27 HARVALD B HANEL, K H SQUIRES R, and TRAP-JENSON J Adenosine-triphosphatase deficiency in patients with non spherocytic haemolytic anaemia Lancet ii 18–19 (1964)
28 JENSSON O JONASSON T, and OLAFSSON O Hereditary elliptocytosis in Iceland Brit J Haemat 13 844–854 (1967)
29 KORNBERG A and HORECKER B L Glucose-6-phosphate dehydrogenase in COLOWICK and KAPLAN Methods in enzymology, vol I pp 323–327 (Academic Press, New York 1955)
30 LAMBRECHT K Die Elliptozytose (Ovalozytose) und ihre klinische Bedeutung Ergebn inn Med Kinderheilk 55 295–319 (1938)
31 LEITNER S J Die familiäre Elliptozytose als vererbbare Anomalie der Erythrozyten Dtsch Arch klin Med 183 607–646 (1939)
32 LEWIS S M SZUR L and DACIE J V The pattern of erythrocyte destruction in haemolytic anaemia as studied with radioactive chromium Brit J Haemat 6 122 139 (1960)
33 LING K BYRNE, W L, and LARDY H Phosphohexokinase in COLOWICK and KAPLAN Methods in enzymology vol I pp 306–310 (Academic Press New York 1955)
34 MANDELLI F DERIU L DE LAURENZI A, FONTANA L e PAPA G Comportamento di alcuni enzimi e metaboliti negli eritrociti dei soggetti normali Recenti Progr Med 46 205–231 (1966)
35 MICHOT, F und MARTI H R Nachweis einer erhöhten Glukose-6-Phosphatdehydrogenase Aktivität der Erythrozyten mittels Farbreaktion Klin Wschr 42 1028–1030 (1964)
36 MORGAN E S Co-existing ovalocytosis and hemoglobin C trait U S armed Forces med J 9 1317–1375 (1958)
37 MURPHY J R Erythrocyte metabolism VI-cell shape and the location of cholesterol in the erythrocyte membrane J Lab clin Med 65 756–774 (1965)
38 NENCI G G MIGLIORINI, F SANTTUSANIO F CASAMASSIMA F e GANTERLUNGHI C Microovalocitosi Studio ematologico e genetico di un ceppo familiare con tre membri affetti Haematologica 52 775–813 (1967)
39 OZER L and MILLS, G C Elliptocytosis with haemolytic anaemia Brit J Haemat 10 468–476 (1964)
40 PEARSON A The genetic basis of hereditary elliptocytosis with hemolysis Blood 32 972 978 (1968)
41 PETERS J C ROWLAND M ISRAELS G, and ZIPURSKY A Erythrocyte sodium transport in hereditary elliptocytosis Canad J Physiol Pharmacol 44 817 827 (1966)
42 PIROLA R C Variable expression of hereditary elliptocytosis in one family Med J Austr 49 1015 1017 (1962)
43 PRYOR D S and PITNEY W R Hereditary elliptocytosis. A report of two families from New Guinea Brit J Haemat 13 126–134 (1967)

44 PUXEDDU, A., NENCI, G G; MIGLIORINI, E. e SANTEUSANIO, F Comportamento degli enzimi glicolitici della glutation reduttasi, degli esteri fosforici, dell adenosina e del glutatione ridotto delle emazie, in alcuni stati anemici Haematologica *51* 997–1012 (1966)
45 RACKER, E. Glutathione reductase (liver and yeast), in COLOWICK and KAPLAN Methods in enzymology vol 2, pp 722–725 (Academic Press, New York 1955)
46 REBUCK, J W and VAN SLYCK, E. J An unsuspected ultrastructural fault in human elliptocytes Amer J clin Path. *49* 19–25 (1968)
47 RINGELHANN, B, KONOTEY AHULU, F I D, LEHMANN, H., and LORKIN, P A A Ghanaian adult, homozygous for hereditary persistence of foetal haemoglobin and heterozygous for elliptocytosis Acta haemat., Basel *43* 100–110 (1970)
48 TORLONTANO, G and BOSMAN, C. The haemolytic hypersplenism in hereditary haemolytic diseases Haemat Lat., Milano *11* 3–30 (1968)
49 TORLONTANO, G, TONIETTI, G, CENTURELLI G., and CONTI, C. Incidence, haematological, enzymological findings and haemolysis in the hereditary elliptocytosis. Study of 54 cases. Proc. 10th Int. Congr Haemat., Stockholm 1964 Rass. Fisiopat. clin ter *37* 1–10 (1965)
50 VALENTINE, W N, TANAKA, K. R., and FREDRICKS, R. E. Erythrocyte acid phosphatase in health and disease Amer J clin. Path. *36* 328–332 (1961)
51 VANDEPITTE, J et LOUIS L. L'association elliptocytose-drépanocytose étude de deux familles. Rev Hémat. *10* 19–27 (1955)
52 WROBLESWSKI, E. and LA DUE, J S Lactic dehydrogenase activity in blood Proc Soc exp Biol. Med. *90* 210–213 (1955)

Author's address Dr G TORLONTANO Via Niccoló Piccinni 32, *00199 Roma* (Italy)

Acta Haemat. 48: 12–15 (1972)

Effect of L-Glutaminase on Transformation and DNA Synthesis of Normal Lymphocytes

R. Schrek, K. V. Batra, J. S. Holcenberg, J. Roberts and W. C. Dolowy

Research Service, Veterans Administration Hospital, Hines, Ill.,
Department of Pharmacology, Chicago College of Osteopathic Medicine, Chicago, Ill.
Departments of Medicine and Experimental Animal Medicine, University of Washington, Seattle, Wash.

Abstract. The transformation of lymphocytes by phytohemagglutinin and the uptake of thymidine by DNA are completely inhibited by 1.7 mIU/ml L-glutaminase GA 1.2. The transformation is also inhibited by 1,700 mIU EC2 L-asparaginase/ml, which is estimated to have 34 mIU/ml of L-glutaminase activity. Therefore, glutamine deficiency may be responsible for the inhibition of transformation both by L-glutaminase GA 1.2 and by EC2 L-asparaginase.

Key Words
Asparaginase
DNA synthesis
Glutaminase
Lymphocyte transformation

The transformation of lymphocytes by phytohemagglutinin (PHA) is inhibited by L-asparaginase EC2 [5]. This finding led to the present work, which shows that transformation can be inhibited by another enzyme, L-glutaminase, at a concentration of 0.0017 IU or 1.7 mIU/ml [9].

Method

The transformation of lymphocytes by PHA was measured by counts of the number of lymphoblastoid cells and by the determination of the uptake of ^{3}H-thymidine into DNA. The L-glutaminase was GA 1.2, which had L-asparaginase activity equal to 60% of the glutaminase activity [7]. The lymphocytes were derived from normal blood in 3 experiments and from a tonsil and a lymph node in one experiment each. The number of viable lymphocytes and lymphoblastoid cells was counted before and after 5 days of incubation by means of a slide-chamber method and

Table I Effect of L-glutaminase GA 1.2, asparaginase EC2 and PHA on the survival of normal lymphocytes, on the transformation to lymphoblastoid cells and on the uptake of ^{3}H thymidine into DNA

Enzyme	L-Glutaminase activity mIU/ml	L Asparaginase activity, mIU/ml	PHA, µl/ml	Survival, %	Lymphoblastoid cells, %	DPM/10³ surviving cells
GA 1.2	0	0	0	51 9(5)[1]	1.2(5)	64(4)
GA 1.2	0	0	1	42.5	60.2	1 072
GA 1.2	17	13 6	1	21.5	1 0	26
GA 1.2	5	4 0	1	26 7	16.5	90
GA 1.2	1 7	1 4	1	39 1	31 7	118
EC2	34	1 700	1	37 0(6)	1 0(6)	
EC2	3 4	170	1	42.2	12.4	

[1] Number in parenthesis indicates number of experiments.

phase microscopy The surviving cells (lymphocytes plus lymphoblastoid cells) were expressed as a percentage of the number of lymphocytes in the original suspension before incubation. The percentage of lymphoblastoid cells was based on the number of surviving cells The uptake of thymidine into DNA was expressed as DPM/10³ surviving cells.

Results

According to previous work [8] the addition of 170 mIU glutaminase/ml had little or no cytocidal effect on normal lymphocytes In the present study, 17 mIU completely, and 1 7 mIU enzyme/ml partially, inhibited both the uptake of thymidine and the transformation into lymphoblastoid cells (table I)

To test whether the inhibition was due to the asparaginase activity in the glutaminase preparation, the effect of L-asparaginase EC2 was studied We found that transformation was partially inhibited by 170 mIU L-asparaginase/ml. The finding suggests that the inhibition of transformation was due to the glutaminase and not the asparaginase activity of GA 1 2

In this study, complete inhibition of blastogenesis was produced by 17 mIU of L-glutaminase GA 1.2. Hersh [3] also obtained inhibition with an E coli glutaminase but required 1,000 mIU/ml The E coli enzyme

was a commercial preparation which had a maximum activity at pH 5 and was assayed at this pH, but which had essentially no activity at pH 7 [2]. The glutaminase GA 1 2 of ROBERTS et al [6] was tested for its activity at pH 7 5. The differences in the activity of the enzymes at physiologic pH are probably responsible for the finding that glutaminase GA 1 2 was 60 times as effective in inhibiting blastogenesis as *E coli* glutaminase.

The sensitivity of lymphocyte transformation to very small concentrations of glutaminase raises the question whether inhibition of transformation by the EC2 preparation is due to asparaginase or glutaminase activity. A concentration of 170 mIU asparaginase/ml caused a reduction of transformation. But this concentration contains 3 4 mIU/ml of glutaminase activity, according to CAMPBELL et al [1] and according to our measurements. Both the L-asparaginase and the L-glutaminase activity of EC2 preparations were measured at pH 7 5. In the present study with glutaminase GA 1 2, a concentration of 3 4 mIU/ml would be expected to inhibit transformation. Therefore, the transformation of lymphocytes by L-asparaginase EC2 may be due to the L-glutaminase activity. This hypothesis is in accord with the report of SIMBERKOFF and THOMAS [10] that L-glutamine, but not L-asparagine, reversed the inhibition of lymphocyte transformation by EC2 L-asparaginase.

Similar considerations would apply to the toxic effect of L-asparaginase on leukemic lymphocytes. Previous work [7, 8] has shown that lymphocytes from patients with chronic lymphocytic leukemia are killed when incubated with 170 mIU/ml L-asparaginase EC2 or with 1 7 mIU/ml L-glutaminase GA 1 2. Therefore, we have to consider the possibility that leukemic lymphocytes incubated with EC2 *in vitro* died as a result of glutamine, not asparagine, deficiency. Similarly, KIM et al [4] reported that glutamine activity of *E coli* asparaginase accounted for its inhibition of HeLa cell proliferation.

References

1 CAMPBELL, H A, MASHBURN, L T, BOYSE, E A, and OLD, L J. Two L-asparaginases from *Escherichia coli* B. Their separation, purification and antitumor activity. Biochemistry 6 721–730 (1967)

2 HARTMAN, S C. Glutaminase of *Escherichia coli*. I. Purification and general catalytic properties. J biol Chem *243* 853–863 (1968)

3 HERSH, E M. L-Glutaminase: suppression of lymphocyte blastogenic responses *in vitro*. Science *172* 736–738 (1971)

4 KIM, J H, BOYSE, E A, OLD, L J, and CAMPBELL, H A. Inhibition of HeLa

cell cultures by preparation of *Escherichia coli* L-asparaginase. Biochim. biophys. Acta *185*: 476-479 (1969).
5 Ohno, R. and Hersh, E. M.: The inhibition of lymphocyte blastogenesis by L-asparaginase. Blood *35*: 250-262 (1970).
6 Roberts, J., Holcenberg, J. S., and Dolowy, W. C.: Antineoplastic activity of highly purified bacterial glutaminases. Nature, Lond. *227*: 1136-1137 (1970).
7 Schrek, R. and Dolowy, W. C.: *In vitro* test for sensitivity of leukemic cells to L-asparaginase. Cancer Res. *31*: 523-526 (1971).
8 Schrek, R., Holcenberg, J. S., Roberts, J., and Dolowy, W. C.: *In vitro* cytocidal effect of L-glutaminase on leukaemic lymphocytes. Nature, Lond. *232*: 265 (1971).
9 Schrek, R., Roberts, J., Holcenberg, J. S., and Dolowy, W. C.: *In vitro* effects of L-glutaminase on normal and leukemic cells. Fed. Proc. *30*: 453 (1971).
10 Sharpless, M. S. and Thomas, L.: Reversal by L-glutamine of the inhibition of lymphocyte mitosis caused by *E. coli* asparaginase. Proc. Soc. exp. Biol. Med. *133*: 642-644 (1970).

Authors' addresses: Robert Schrek, M.D., Veterans Administration Hospital, *Hines, IL 60141*; Karam V. Batra, Ph.D., Department of Pharmacology, Chicago College of Osteopathic Medicine, 1122 East 53rd Street, *Chicago, IL 60615*; John S. Holcenberg, M.D., Department of Medicine; Joseph Roberts, M.D., and William C. Dolowy, D.V.M., Department of Experimental Animal Medicine, University of Washington, *Seattle, WA 98105* (USA)

Acta Haemat. 48: 16–23 (1972)

Beeinflussung der Nukleinsäuresynthese menschlicher Granulozyten durch Dexamethasonhemisulfat

H. G. Rainer, K. Moser, J. Schwarzmeier

I. Medizinische Universitätsklinik Wien (Vorstand: Prof. Dr. E. Deutsch)

Abstract. Dexamethasone hemisulphate (Dexa-Scheroson®) in concentrations of 100 μg/ml culture medium produces a marked inhibition of the incorporation of ¹⁴C-adenine into the trichloroacetic acid precipitate and nucleic acids of granulocytes. Exact analysis of the precursor pool and measurement of the rate of nucleic acid synthesis showed that this inhibition of incorporation by the dexamethasone ester was due to a reduction in the formation of fresh nucleic acids. Fractionation of these extracts in linear sucrose gradients revealed a remarkable diminution in the radioactivity of the fractions 18 S and 4 S. We believe the cause of this change in metabolism could be that a false ribosomal RNA precursor is formed which does not contain the 18 S portion. Alternatively, one might postulate an increased instability of this component. Such changes could lead to a reduction in existing ribosomes and in protein synthesis. In this case, the diminished turnover of transfer nucleic acids perhaps corresponding to the component sedimenting at 4 S could be interpreted as a further rational biological reaction.

Key Words
Dexamethasone
Glucocorticoids
Leukocyte nucleic acids
Nucleic acid metabolism
Protein synthesis of leukocytes

Der katabole Effekt von Glukokortikoiden auf Zellen des lymphatischen Systems [3–7, 10, 11] und der myeloischen Reihe kann durch zahlreiche Arbeiten [13, 14] belegt werden. Drews [3, 4], Brawerman [5] und Drings *et al.* [6] konnten zeigen, dass *in vivo* bzw. *in vitro* appliziertes Prednisolon in Thymuszellen und Phytohämagglutinin-stimulierten Lymphozyten vorwiegend die Neusynthese derjenigen RNS-Komponente hemmt, die dazu bestimmt ist, struktureller Bestandteil der Ribosomen zu werden.

Über die Nukleinsäure- und Proteinsynthese von Granulozyten liegen nur die Untersuchungen von WERTHAMER und AMARAL [13] und WERTHAMER *et al* [14] vor, die mit der Autoradiographie zeigen konnten, dass Granulozyten nach Cortisolvorbehandlung eine verminderte Inkorporation von ^{3}H Uridin aufweisen

Wir haben geprüft, ob die mit der Autoradiographie gefundene Inkorporationshemmung tatsächlich einer verminderten Nukleinsäuresynthese entspricht Weiters sollte getestet werden, ob das Sedimentationsmuster der schnell markierten RNS-Anteile in einem linearen Sucrosegradienten demjenigen unbehandelter Zellen entspricht.

Methodik

Inkubation von Granulozyten mit ^{14}C Adenin Für die Blutabnahme wurden gesunde Personen herangezogen 100 ml Blut wurden mit 0,5 ml Liquemin Roche® und 20 ml Dextranlösung (5%, MG ca. 204 000) versetzt, die Erythrozyten sedimentiert und aus dem Plasmaüberstand die Granulozyten [12] isoliert.

Die Granulozyten, die mit maximal 10% Lymphozyten kontaminiert waren, wurden mit 2 ml TC-199 Medium (mit Penizillin und Streptomyzinzusatz) und 100 μg Dexamethasonhemisulfat (Dexa Scheroson®, Schering AG)/ml TC-199 Medium 60 min hindurch im Schüttelwasserbad bei 37 °C inkubiert Kontrollproben erhielten keinen Zusatz. Im Anschluss wurden die Leukozyten mit 5 μCi ^{14}C-Adenin (Radiochemical Centre, Amersham spezifische Aktivität 430 mCi/mg) pro 2 ml Kulturmedium versetzt Die Inkubationszeit betrug 60 min Für kinetische Untersuchungen wurden kürzere Inkubationszeiten gewählt.

Bestimmung der Trichloressigsäure (TCA)-unlöslichen Aktivität Nach Abschluss der Inkubationsperioden wurden die Zellen mit eiskalter Phosphatpufferlösung [12] versetzt, 5 min bei 200 *g* bei 0–4 °C zentrifugiert, der Überstand verworfen und das Zellpellet einer gleichen zweiten Waschung unterworfen Anschliessend wurden die Zellen in 5 ml Acetatpuffer (0 01 M pH 5 1, 0,1 M NaCl 0 001 MgCl, 1% Bentonit 0,5% Sodiumdodecylsulfat resuspendiert und im Gerät nach Potter Elvehjem homogenisiert. 0 1 ml des Homogenates wurden auf ein 2×2 cm messendes Whatman 3 MM Filterpapier pipettiert und 0 02 ml für die Proteinbestimmung nach Lowry bereitgestellt.

Die Filterblättchen wurden den folgenden Waschprozeduren unterworfen 10% TCA, Äthanol + 10% K Acetat, 5% TCA, Äthanol, Chloroform/Methanol (2 1) Äther

Nach dem Trocknen wurde die Aktivität im Tri Carb (Packard) bestimmt

Extraktion der Nukleinsäuren bei 37 °C (warm extraction technique [1, 2]). Zu den in Acetatpuffer (siehe oben) suspendierten Leukozyten wurden 5 ml redestillierte, wassergesättigte Phenollösung (500 ml Phenol, 70 ml Kresol aq bidest. q s., 0,1% 8-Hydroxychinolin) zugegeben und 2 min homogenisiert. Durch scharfes Zentrifugieren wurden wässerige und Phenolphase getrennt und die Nukleinsäuren

durch Zusatz von $2^1/_2$ Vol Äthanol + 10% K Acetat aus der wässerigen Phase ausgefällt. Nach Lösung der Nukleinsäuren wurde die spezifische Aktivität dieser Verbindung durch Liquidszintillationszählung und Absorptionsmessung am Photometer Zeiss PM Q II (260 mμ) ermittelt

Bestimmung der spezifischen intrazellulären Adeninaktivität aus den Adeninnukleotiden (Precursorpoolaktivität) Die Nukleotide wurden an DOWEX absorbiert, gereinigt [8], hydrolysiert und papierchromatographisch [8] die spezifische Adeninaktivität ermittelt

Fraktionierung der extrahierten Nukleinsäuren mittels der Gradientenzentrifugation Auf lineare Sucrosegradienten [6] wurden 50–150 μg RNS aufgeschichtet und die Trennung der Nukleinsäurefraktionen wie bei DRIVOS *et al* beschrieben [6] durchgeführt Die einzelnen Fraktionen wurden durch Anstechen und Austropfen der Röhrchen gesammelt

Die Auswertung dieser Ergebnisse erfolgte durch eine dem Sedimentationsmuster entsprechende Unterteilung der Kurve in 4 Bereiche und Kurvenintegration durch ein Näherungsverfahren

Ergebnisse

Die Inkorporation von ^{14}C-Adenin nimmt während der Inkubationsdauer von 60 min in das TCA-unlösliche Präzipitat und in die extrahierten Nukleinsäuren nahezu linear zu Bei mit Dexamethasonhemisulfat vorbehandelter Granulozytensuspensionen ist diese Aktivitätszunahme signifikant verringert (Tab I)

Allerdings konnten durch ein verändertes Angebot des radioaktiv markierten Vorläufers auch fälschlicherweise verminderte Syntheseraten vorgetäuscht werden Um auszuschliessen, dass die ermittelte Abnahme der Einbaurate von Adenin nicht nur einer Verkleinerung des radioaktiven Precursorpools entspricht, haben wir die Extraktaktivität und die Precursorpoolaktivität behandelter und unbehandelter Leukozytenkulturen gemessen Die Extraktaktivität liegt dabei in vorbehandelten Kulturen unbedeutend höher, die spezifische Aktivität von Adenin ist in den säurelöslichen Adeninnukleotiden etwas verringert (Tab II) Die Unterschiede sind nicht signifikant

Die Berechnung relativer Syntheseraten (Nukleinsäureaktivität Extraktaktivität) ergab eine signifikante Verringerung der Nukleinsäuresyntheseraten bei mit Dexamethasonhemisulfat vorbehandelten Granulozyten (Tab III)

Die Fraktionierung der extrahierten Nukleinsäuren im linearen Sucrosegradienten (5–20%) ergab verschiedene Sedimentationsmuster in Versuchs- und Kontrollansätzen Zur statistischen Auswertung der Er-

Tabelle I Inkorporation von ^{14}C-Adenin während einer Inkubationsdauer von 1 h in das säurelösliche Präzipitat und in Nukleinsäuren (extrahiert bei 37°C, pH 5 1) von Granulozyten Normwerte und Beeinflussung der Inkorporation durch Zusatz von 100 µg Dexamethasonhemisulfat/ml Medium (Mittelwerte und Standardabweichungen) n = [illegible]

	dpm/mg Protein	
	Mittelwert	Standardabweichung
Aktivität des TCA unlöslichen Präzipitates		
TCM Medium	5 214	± 1 453
TCM Medium + Dexamethasonhemisulfat	2 513	± 1 [illegible]
	dpm/µg	
	Mittelwert	Standardabweichung
Spezifische Aktivität der Nukleinsäuren		
TCM Medium	76,30	35 7
TCM Medium + Dexamethasonhemisulfat	23 15	± 5 09[1]

[1] Statistisch signifikant nach t Test

Tabelle II Beeinflussung der Extraktaktivität und der spezifischen Aktivität von Adenin aus den säurelöslichen Adeninnukleotiden durch Zusatz von 100 µg Dexamethasonhemisulfat/ml Granulozytensuspension (Mittelwerte und Standardabweichungen) n = 5

	dpm/mg RNS	
	Mittelwert	Standardabweichung
Extraktaktivität		
TCM Medium	[illegible]	± [illegible]
TCM Medium + Dexamethasonhemisulfat	[illegible]	± [illegible]
	dpm/mg	
	Mittelwert	Standardabweichung
Spezifische Adeninaktivität		
TCM Medium	[illegible] 000	± [illegible] 000
TCM Medium + Dexamethasonhemisulfat	6 [illegible] 000	± [illegible] 624 000

gebnisse wurden die [illegible] in 4 Flächen unterteilt und aus den Flächen[illegible] [illegible] Anteile der einzelnen Nukleinsäuren ermittelt. Der [illegible] dieser [illegible] Transfernukleinsäuren mit er-

Tabelle III Beeinflussung der relativen Syntheseraten (Nukleinsäureaktivität/Extraktaktivität) in Granulozyten durch *In-vitro*-Zusatz von 100 µg Dexamethasonhemisulfat/ml Medium (Mittelwerte und Standardabweichungen)

	Mittelwert	Standardabweichung
TCM-Medium	0,5102	±0,2755
TCM-Medium + Dexamethasonhemisulfat	0,1358	±0 0889

Tabelle IV. Beeinflussung des Sedimentationsmusters des 18 S- und 4 S Peak im linearen Sucrosegradienten (5–20%) von Nukleinsäuren aus Granulozytensuspensionen durch *In-vitro*-Zusatz von 100 µg Dexamethasonhemisulfat/ml Durch Kurvenintegration der OD 260 nm und der Aktivitäten der einzelnen durch Austropfen gesammelten Fraktionen wurden die spezifischen Aktivitäten der beiden Bereiche ermittelt (Mittelwerte und Standardabweichungen) n = 3

	dpm/µg RNS 18 S-Bereich		dpm/µg RNS 4 S-Bereich	
	Mittelwert	Standardabweichung	Mittelwert	Standardabweichung
TCM-Medium	22,6	±6,2	35,3	±7,9
TCM-Medium + Dexamethasonhemisulfat	5,4	±2,3[1]	14,7	±5,0[1]

[1] Statistisch signifikant nach t-Test

ner Sedimentationskonstanten von 4 S entsprechen der 2 Teil beinhaltet ribosomale Ribonukleinsauren mit einer Sedimentationskonstanten von 18 S, der 3 Teil solche mit 28 S und der 4 Bereich hohermolekulare Nukleinsauren Wie aus Tabelle IV ersichtlich wird, nimmt durch Dexamethasonhemisulfat die Markierung des 4 S- und 18 S-Bereiches ab

Bei einer Patientin mit idiopathischer Thrombozytopenie haben wir vor und nach Dexamethasonhemisulfatbehandlung (Dexa-Scheroson® 25 mg/die, durch 7 d hindurch) Inkorporationsstudien an den Granulozyten vorgenommen, die prinzipiell die *in vitro* gefundenen Ergebnisse bestatigten Die Hemmung der Neuproduktion von Nukleinsauren erscheint jedoch nach Behandlung noch ausgeprägter (Tab V)

Tabelle 1. Beeinflussung der Inkorporation von 14C-Adenin in die Nukleinsäuren von Granulozyten durch 2tägige Applikation von 25 mg die Dexamethasonberusulfat i.m.

	Vor Dexamethason-berusulfat-behandlung	Nach Dexamethason-berusulfat-behandlung
TCA-unlösliches Präzipitat, dpm/mg Protein	6 100	3 359
Nukleinsäureaktivität dpm/μg RNS	97,9	12,0
Extraktaktivität, dpm/mg RNS	233 933	[illegible]
Relative Syntheseraten	0,46	[illegible]
Spezifische Aktivität des 18 S-Anteils, dpm/μg	31 447	0,4
4 S-Anteils, dpm/μg	39 335	[illegible]

Diskussion

Unsere Untersuchungen der Nukleinsäuresynthese in menschlichen Granulozyten nach Kortikoidzusatz *in vivo* konnten die autoradiographischen Befunde [13] bestätigen. Verfeinerte biochemische Methoden haben es uns dabei erlaubt, über den Mechanismus der [illegible] Produktionseinschränkung nach Behandlung und Zusatz [illegible] von Dexamethasonberusulfat zusätzliche Aussagen zu machen.

Die Verminderung des Radioaktivitätspeaks im 18 S-Bereich [illegible] darauf beruhen, dass ein rudimentärer ribosomaler RNS-Vorläufer gebildet wird, der das später abzuspaltende 18 S-Teilstück nicht enthält. Andererseits könnte der Mangel dieser Komponente auch durch eine besondere Instabilität dieses Anteils entstehen.

Normalerweise produziert der reife Lympho- und Granulozyt nur so viele ribosomale RNS, als zur Aufrechterhaltung der normalen Anzahl an Ribosomen notwendig ist. [illegible] maler RNS wurden von [illegible] [5] im Zusammenhang mit Zellwachstum und [illegible] gefunden. [illegible] Fehlen der 18S-Komponente [illegible] [2] in Blutzellen als Ausdruck [illegible] eines [illegible]

standes gedeutet, der durch fehlende oder sehr geringe Neubildung von Ribosomen gekennzeichnet ist.

In gleicher Weise konnte als «biologischer Ruhezustand» auch die unter Dexamethason beobachtete Verminderung der spezifischen Aktivitat des 4S-Bereiches in Granulozyten gedeutet werden. Da eine verminderte Neuproduktion von Ribosomen auftritt, ist es denkbar, dass die Proteinsynthese absinkt. Es ware dabei durchaus im Sinne einer sinnvollen biologischen Reaktion, wenn in gleicher Weise der «turnover» der 4 S-RNS abnimmt, da dieser Anteil den Transfer-RNS entsprechen könnte.

Unsere Ergebnisse weisen bemerkenswerte Parallelen zu den Untersuchungen von DREWS [3, 4], BRAWERMANN [5] und DRINGS *et al.* [6] auf. Diese Analogie lasst vermuten, dass prinzipiell dasselbe regulatorische Prinzip fur die Stoffwechselveranderungen in Lymphozyten, Thymozyten und Granulozyten fur die Verminderung der RNS-Synthese verantwortlich erscheint. In allen Fällen kommt es zu einer Verminderung der Neubildung ribosomaler Vorstufen, wobei das Sedimentationsmuster eine stark verminderte, bei 18 S sedimentierende Komponente aufweist.

Zusammenfassung

Dexamethasonhemisulfat (Dexa-Scheroson®) bewirkt in Konzentrationen von 100 μg/ml Kulturmedium eine deutliche Hemmung der ^{14}C-Adenininkorporation in das Trichloressigsäure fällbare Präzipitat sowie in die Nukleinsäuren von Granulozyten Eine Analyse des Precursorpools und der Nukleinsäuresynthese in den Zellen ergab, dass diese Inkorporationshemmung durch Verminderung der Neuproduktion von Nukleinsäuren bedingt ist Die Fraktionierung dieser extrahierten Verbindungen im linearen Sucrosegradienten ergab eine bemerkenswerte Verminderung der Radioaktivität der 18 S- und 4 S-Fraktion Die möglichen Ursachen dieser metabolischen Veränderung werden diskutiert

Literatur

1 COOPER, H L. Ribosomal ribonucleic acid production and growth regulation in human lymphocytes J biol Chem *244* 7 (1969)

2 COOPER, H L. and KAY, J E. Differential extraction of nuclear and cytoplasmic RNA from intact lymphocytes. Biochim biophys Acta *174* 503 (1969)

3 DREWS, J The effect of prednisolone injected *in vivo* on RNA synthesis in rat thymus cells. Europ J Biochem. *7* 200 (1969)

4 DREWS, J The effect of prednisolone injected *in vivo* on RNA polymerase activities in isolated rat thymus nuclei. Europ J Biochem. *13* 231 (1970)
5 BRAWERMAN, G Alteration in the nature of ribonucleic acid synthetized in rat liver during regeneration and after cortisol administration J biol Chem. *242* 801 (1967)
6 DRINGS, P, FÖLSCH, E., und DREWS, J Die Wirkung von Prednisolon auf die Synthese von RNS und DNS in Phytohamagglutinin stimulierten Lymphozyten. Z. ges. exp Med *153* 35 (1970)
7 MAKMAN, M H, DVORKIN, B., and WHITE, A Alterations in protein and nucleic acid metabolism of thymocytes produced by adrenal steroids *in vitro* J biol Chem. *241* 1544 (1966)
8 MARKO, P, GERLACH, E., ZIMMER, H G, PECHAN, I, CREMER, T., and TRENDELENBURG, C. Interrelationship between salvage pathway and synthesis *de novo* of adenine nucleotides in kidney slices. Z. Physiol Chem *350* 1669 (1969)
9 NOWELL, P C Inhibition of human leucocyte mitosis by prednisolone *in vitro* Cancer Res *21* 1518 (1961)
10 ONO, T, TERAYAMA, H, TAKAKU, F, and NAKAO, K. Inhibitory effects of hydrocortisone upon the phytohemagglutinin induced RNA synthesis in human lymphocytes Biophys Acta *161* 361 (1968)
11 PENAN, A., DVORKIN, B., and WHITE, A Acute effect of a single *in vivo* injection of cortisol on *in vitro* amino acid incorporation activity of rat liver and thymic preparations Biochem biophys Res Comm *16* 449 (1964)
12 RAINER, H., SCHWARZMEIER, J, SCHNACK, H., und MOSER, K. Granulocyten stoffwechsel bei Patienten mit Porphyria cutanea tarda Wien klin. Wschr (in Druck)
13 WERTHAMER, S and AMARAL, L. The effect of cortisol on the incorporation of RNA and protein precursors into the nucleus and cytoplasma of human neu-

Adresse der Autoren Dr H G RAINER, Doz. Dr K. MOSER und Dr J SCHWARZMEIER, I Medizinische Universitätsklinik, Lazarettgasse 14, *A-1090 Wien* (Österreich)

Acta Haemat 48 24-27 (1972)

The Effect of Serum Protein Fractions of the Newborn on the Thrombocytopoiesis of Mice

F Krizsa, Z. Kovács, E Dobay and L. Varga

I Medical Clinic, Clinic of Sick Children and II Medical Clinic of the Medical University, Szeged

Abstract The effect on thrombocytopoiesis in mice of serum, respectively serum protein fractions of 3 healthy infants and of 5 hyperbilirubinemic infants treated with exchange transfusion was studied Normal infant serum does not influence the number of circulating thrombocytes in mice The β fraction of normal infant serum as well as the whole serum, its β and to a lesser degree α_1 fraction of serum obtained after exchange transfusion show a thrombocyte increasing effect

Key Words
Exchange transfusion
Neonatal serum proteins
Thrombocytosis
Thrombopoietic factors

Dux *et al* [1, 2] studied the changes in thrombocyte count after neonatal exchange transfusion, and the humoral relation of these changes Serum samples taken 24–48 h after exchange transfusion caused thrombocytosis in mice with a maximum on the fifth day In a previous investigation [5] we showed that serum taken after exchange transfusion produces an increase of giant cells in the bone marrow and spleen of mice and a shift to the left in the megakaryocyte system From this we concluded that the factor studied is a substance of thrombopoietic effect

In our present series of experiments we studied the effect of electrophoretically separated serum protein fractions of infants, who received exchange transfusion, on the thrombocytopoiesis of mice

Material and Method

We studied the serum protein fractions obtained 24–48 h after exchange transfusion from 5 infants treated for neonatal hyperbilirubinemia and of 3 healthy in

fants The determinations were carried out on 3- to 4-month-old mice with a body weight of 20 g of the strain BALB/c and receiving standard food The average thrombocyte count of 20 untreated mice was 851 000 the SD ± 118 000 The thrombocytes were counted with the direct phasecontrast microscopic method described by FISCHER and GERMER [3]

For the determination of thrombopoietic activity of serum, or of protein fractions 5 mice of each group received intraperitoneally 0 4 ml of the substance under study after the preliminary determination of the circulating thrombocyte count On the basis of previous experiences and theoretical reasoning the fifth day was selected for the evaluation of the effect.

Electrophoresis was carried out on an agar gel medium For this purpose we used the 1% Difco agar in a veronal buffer of pH 8 6 and 0 05 ionic concentration Electrophoresis lasted 15 h at 50 mA. Protein was isolated from the fractions through a porcelain filter in vacuum

The protein fractions were controlled with immune electrophoresis, according to the technique of SCHEIDEGGER [8] and the polyvalent horse serum labelled 249 of the Human Works was used as precipitating serum.

For the determination of significance we used the Student t test.

Results

The serum of healthy infants did not change the thrombocyte count in mice The serum obtained 24–48 h after exchange transfusion resulted on the fifth day in a significant increase of thrombocytes All serum fractions caused an increase in the number of platelets, but the effect of fraction α_1 and β of the serum obtained after exchange transfusion, as well as the fraction β of healthy children was the most significant, the change caused by other fractions was within the range of normal oscillation and of mis calculation (fig 1)

The sera, or serum fractions of healthy children and those receiving exchange transfusion showed no quantitative or qualitative differences on immune-electrophoresis and polyacrylamide gel electrophoresis

Discussion

KELEMEN *et al* [4] were the first who reported data on the plasma factor (thrombopoietine) regulating humorally the platelet formation Ac-

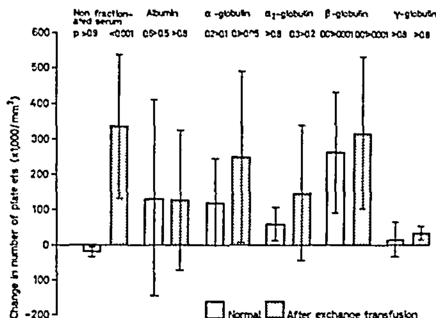


Fig 1 The effect of serum fractions of healthy infants and of infants having received an exchange transfusion on the thrombocyte count of mice Changes in thrombocyte count observed on the fifth day are presented The columns represent the values obtained in 5 mice of each group with normal infant serum (in total 15 mice) and in 5 mice (25 in total) with serum obtained of infants, who had undergone exchange transfusion The vertical lines show the SD values

cording to their investigations it is a thermo- and pH sensitive substance migrating with the β globulin fraction In rabbits STEINBERG *et al* [10] observed the thrombocyte increasing effect of human albumin

The thrombocytosis producing activity of human serum was localized by SCHULMAN *et al* [9] in the α_2 globulin fraction RÁK *et al* [7] observed *in mice the thrombocytosis producing effect of the β globulin fraction of* normal human serum

The different results of individual teams are still difficult to interpret at present, the differences being probably due to various methods of examination

As in the investigations of RÁK *et al* [7] the β globulin fraction of the serum of healthy infants showed in our series of experiments a thrombocyte increasing effect, but the whole serum did not cause a rise of the thrombocyte count It is most probable that some inhibitory substance of the normal serum prevents the action of the factor migrating with the β globulin fraction

Serum obtained after exchange transfusion produces thrombocytosis, of the serum fractions the β and in a lesser degree the α_1 show a thrombocyte increasing action The methods used seem not to be suitable to decide whether there are any qualitative or quantitative differences between the individual fractions of normal serum and of that obtained after exchange transfusion The detailed analysis of the possible inhibitory factor of normal serum, or of the possible lack or decrease of inhibitor in thrombopoietically active sera may have fair prospects

References

1 Dux E. Kovács Z. und Gömesy, F Beiträge zur Frage der humoralen Regulation der Thrombopoese Untersuchungen über die im Anschluss an Austauschtransfusionen bei Neugeborenen auftretende Thrombopenie Acta haemat., Basel *27* 334 (1962)

2 Dux, E., Kovács Z. und Pataki L. D e Änderung der Thrombocytenzahl nach Austauschtransfusionen bei Neugeborenen. Mschr Kinderheilk. *111* 128 (1963)

3 Fischer W und Germer W D Über eine einfache Methode der direkten phasenoptischen Thrombozytenzählung Rontgen Lab Praxis *10* 49 (1957)

4 Kelemen E. Cserháti, I., and Tanos B Demonstration and some properties of human thrombopoietin in thrombocythaemic sera Acta haemat., Basel *20* 350 (1958)

5 Krizsa F, Kovács, Z., and Dobay E Effect of exchange transfusion induced thrombocytopoietic activity of infants sera on the megakaryocytic system of mice Haematologia *3* 237 (1969)

6 Ornstein L. Disc electrophoresis I Background and theory Ann N Y Acad. Sci. *121* 321 (1964)

7 Rák, K. Varga, L. Krizsa F und Cserháti, I Die Wirkung von Eiweissfraktionen normaler menschlicher Sera auf die Thrombocytenzahl der Maus. Experientia *19* 136 (1963)

8 Scheidegger, J J Une microméthode de l'immunoéléctrophorèse. Int Arch. Allergy *7* 103 (1956)

9 Schulman I Pierce, M, Lukens A., and Currimbhoy Z Studies on thrombopoiesis I A factor in normal human plasma required for platelet production chronic thrombocytopenia due to its deficiency Blood *16* 943 (1960)

10 Steinberg B Dietz, A. A., and Martin R. A. Mechanism of hematopoiesis Hemacytopoietic factors in human plasma. Acta haemat., Basel 21 78 (1959)

Authors address Dr F Krizsa, Dr Z Kovács Dr E. Dobay and Dr L Varga, I Department of Medicine University Medical School Korányi RkP 12, *Szeged* (Hungary)

Acta Haemat. 48 28-33 (1972)

Early Splenectomy in the Management of Thalassemic Children in Djakarta[1]

I Wahidiyat, A H Markum and Z. K Adang

Division of Hematology and Child Surgery, Department of Child Health Medical School University of Indonesia Djakarta

Abstract A comparison was made between the results of splenectomy performed on 19 children with thalassemia major, who were operated on after the signs of hypersplenism and hemosiderosis appeared and on 25 children (11 with thalassemia Hb E disease and 14 with thalassemia major) who were splenectomized on indication of increased blood requirement only, before signs of hypersplenism or hemosiderosis appeared

From the results it can be concluded that splenectomy should be performed as soon as increased transfusion requirement appears, before hypersplenism or hemosiderosis becomes apparent Due to high frequency of postsplenectomy infections, it is still preferable to operate the child after 2 years of age

Key Words
Haemosiderosis
Hb E disease
Hypersplenism
Splenectomy in thalassemia
Thalassemia

Transfusion therapy on a continuing basis remains the major form of treatment for patients with Cooley's anemia. On the other hand, excessive blood transfusions cause iron overload which may deteriorate the condition of the child [16] These high requirements of blood transfusions are usually due to increased hemolysis The spleen is one of the sites of red-cell destruction and causes hypersplenism [19] Splenectomy may reduce such disadvantageous conditions and therefore may prolong the life of those children when it is performed at the proper moment On the other hand, especially in younger children, the spleen is still an important organ in the defence mechanism [6] Gross [8] concluded that the spleen has its maximum function during the first years of life Splenectomy performed on young thalassemic children will increase their susceptibility to infections [10 14 17, 18] But on the other hand, hemosiderosis and hemo-

[1] This paper was presented at the 2nd Meeting of the Asian Pacific Division of The International Society of Haematology Melbourne May 24th-28th 1971

chromatosis of tissue cells, which are usually found in advanced cases of thalassemia, may cause functional disturbances of various organs [5] The indication for splenectomy in Cooley's anemia is still in debate and may be different in various centers The majority are of the opinion that the major indications are increased blood transfusion requirements, signs of hypersplenism (pancytopenia) and large size of the spleen, which causes discomfort to the patient [2] Our experience of splenectomy performed on 19 children, based on the above signs and symptoms, did not show any satisfactory result

In the last 4 years, we have tried to remove the spleen of thalassemic children before the presence of hypersplenism or excessive iron deposits in the tissues Due to the high incidence of infections, we prefer to do it after the age of 2 years [12] The purpose of this paper is to report our experience and to try to establish the most proper moment for performing splenectomy on children with thalassemia

Material and Methods

Our material consisted of 25 children (group A) varying in age from 4 to 17 months at the time diagnosis was established later operated on at an early stage, and a comparison group of 19 children (group B 3–36 months of age at the time of diagnosis) operated on after the signs of hypersplenism or/and hemosiderosis appeared By early stage is meant the stage at which the child was still in good condition, without any signs of hypersplenism (pancytopenia), hemosiderosis or hemochromatosis. The only indication of splenectomy in group A was the increased blood transfusion requirements (the interval of transfusions becomes shorter from 3–4 months to 3–4 weeks) Hemosiderosis was detected by the presence of large amounts of hemosiderin granules in the bone marrow, stained with Prussian blue 13 of the 25 group-A children were operated on during the last 4 years, and the other 12 cases were selected from the medical records. Routine blood examinations, including hemoglobin determination, reticulocytes, white cells, differential count [illegible]

[illegible]

um iron and iron binding capacity were performed by the method of HAGBERG [9]

Results

All 25 children from group A showed an increased concentration of Hb F (17 3–80 8%) 11 of the group revealed the presence of Hb E on electrophoresis (thalassemia Hb E disease), the remaining 14 children had thalassemia major Diagnosis of the 14 children was established at

Table I Data on splenectomy

Group		Number of cases	Average age at diagnosis, months	Average age at operation, months
A	Thal major	14	15 (4–17)	40 8 (18–77)
	Thal Hb E disease	11	69 5 (18–151)	92.5 (54–156)
B	Thal major	19	13 2 (3–36)	54 (18–116)

Table II Transfusion requirement/year

Group		Splenectomy	
		before	after
A	Thal major	±4 series	±1 3 series
	Thal -Hb E disease	±1.5 series	0
B	Thal major	8–12 series	±6 series

the average age of 15 months, whereas for the thalassemia-Hb E group, it was at 68 5 months The average age at which splenectomy of the former group was performed was 40 8 (18–77) months for pure thalassemia major and 92 5 (54–156) months for the thalassemia-Hb E disease (table I) The average transfusion requirements before splenectomy in children with thalassemia major were about 4 series a year and 1 5 for thalassemia Hb E disease After the operation, these requirements decreased to 1 3 and 0 series a year for thalassemia major and thalassemia-Hb E disease, respectively (table II) Usually, 1 series of transfusions consisted of 2–3 units of blood Except for one case, all children with thalassemia Hb E disease needed no more blood transfusions following the operation In 21 children of this group, the serum iron was increased (142 2–281 2 μg%), with a low iron-binding capacity (0–50 μg%) Only 2 children from group A died during a 14- to 118-month follow-up (table III), one of them suffering from thalassemia-Hb E disease

All 19 children in group B had pure thalassemia major and were operated on at the average age of 54 (18–116) months (table I), while the average age at which diagnosis was confirmed was 13 2 months Before the spleen was removed, nearly all the children needed blood transfusion ev-

Table III Follow-up

Group		Number of cases	Fatal outcome	Duration of follow-up after splenectomy, months
A	Thal major	14	1	68.5 (24-188)
	Thal. Hb E disease	11	1	52.3 (14-114)
B	Thal major	19	12	22.4 (0-70)

ery month, after the operation, this requirement still remained high. All children in this group showed large amounts of hemosiderin granules in the bone marrow 12 of the 19 children died from severe infections within 20 9 (0-70) months following operation. Four of them died within several days after splenectomy, accompanied by high fever (sepsis?) The main cause of death in this group was bronchopneumonia, in some, it was associated with heart failure

Discussion

In managing children with thalassemia, we very often have to face conditions where splenectomy cannot be avoided, although it is known that splenectomy may be followed by hazardous infections with a fatal outcome 19 children splenectomized with hypersplenism and/or hemosiderosis did not show any improvement, and the condition even deteriorated 12 out of the 19 group-B children died within 20 9 (0-70) months following the operation, whereas 4 of them died only within several days post splenectomy, accompanied by high fever These severe infections appeared usually within 2 years after the spleen was removed [15, 18] The other 7 children showed only little improvement after the operation and the transfusion requirement still remained high. This unsatisfactory result was most probably caused by excessive iron deposits in the organs, producing *disturbances in their functions* Large amounts of hemosiderin granules were found in the bone marrow of the group-B children On the other hand, splenectomy performed on the 25 group-A children, who did not yet show any sign of hemosiderosis or *hypersplenism (pancytopenia)*, revealed better results

After splenectomy, 14 children with thalassemia major (from group A) still needed blood transfusions, but with much less frequency. Before the spleen was removed, the children needed 3–4 series of blood transfusions in a year as against only 0–2 series after the operation. During the follow-up of 46–84 months post splenectomy, 3 of the 14 children no longer needed blood transfusion. Only 2 of the 25 group-A children expired, within 14- and 105-month follow-ups, respectively. One of them suffered from thalassemia Hb E disease. The cause of death in these 2 children was bronchopneumonia and purulent meningitis.

Patients with thalassemia-Hb E disease came usually at an older age to the doctor with complaints of distended abdomen and/or moderate anemia. They usually showed milder symptoms than those of thalassemia major. Most of these patients did not require anymore blood transfusions during the follow-up of 52.3 (14–186) months post splenectomy, although they were operated on at an older age (92.5 months). If we compare the results of the operations of the 14 thalassemic children from group A, who were operated on in very good condition, with those of the other 19 children from group B who were operated on after signs of hypersplenism and hemosiderosis appeared, it is evident that the first group of children had a more hopeful future than the latter. The most important problem in planning splenectomy in children with thalassemia is to decide the most proper moment to perform the operation. Various authors, such as ROBINSON and STURGEON [15], did not find any correlation between the high incidence of infections following operation and the age of splenectomy. When it is feasible, the authors prefer to perform splenectomy on older children (more than 2 years of age), provided that there is no sign of hypersplenism or iron over loading. But splenectomy should not be postponed if there are increased transfusion requirements, even when the child is below 2 years of age. Splenectomy done on healthy and older children who suffer from traumatic spleen rupture is seldom followed by overwhelming infections [7, 11, 13, 15]. Next to splenectomy, administration of desferrioxamin and high level packed cell transfusions [20] may prolong the life of children with thalassemia. However, some authors [3] do not see any benefit of high level transfusions.

References

1 BETKE, K., MARTI, H. R., and SCHLICHT, I.: Estimation of small percentage of foetal hemoglobin. Nature, Lond. *184:* 1877 (1959).

2 BOURONCLE, B A and DOAN, C A Cooley's anemia, indications for splenectomy Ann N Y Acad Sci *119* 709 (1964)
3 BROOK, C. G D, THOMPSON, E N, MARSHALL, W C., and WHITEHOUSE, R H Growth in children with thalassemia major and effect of two different transfusion regimens Arch Dis Childh *44* 612 (1969)
4 DACIE, J V and LEWIS, S M Practical hematology, 3rd ed (Churchill, London 1963)
5 ELLIS, J T, SCHULMAN, I, and SMITH, C. H Generalized siderosis with fibrosis of liver and pancreas in Cooley's (Mediterranean) anemia, with observations on pathogenesis of siderosis and fibrosis Amer J Path *30* 287 (1954)
6 ELLIS, E F and SMITH, R T The role of the spleen in immunity, with special reference to the post splenectomy problem in infants Pediatrics *37* 111 (1966)
7 GOFSTEIN, R and GELLIS, S S Splenectomy in infancy and childhood The question of overwhelming infection following operation Amer J Dis Child *91* 566 (1956)
8 GROSS, L. Studies on the gross and minute anatomy of the spleen in health and disease J med Res *39* 311 (1919), taken from Ann Surg *136* 239 (1952)
9 HAGBERG, B The iron binding capacity of serum in infants and children Acta paediat *42* suppl, vol 93 (1953)
10 HORAN, M B and COLEBATCH, J H Relation between splenectomy and subsequent infection Arch Dis Childh *37* 398 (1962)
[illegible]
on children with thalassemia Paediat indones 9 29 (1969)
13 [illegible]
14 [illegible]
15 ROBINSON, T W and STURGEON, P Post splenectomy infection in infant and children Pediatrics *25* 491 (1960)
16 SCHORR, J B and RADEL, E Transfusion therapy and its complications in patients with Cooley's anemia Ann N Y Acad Sci *119* 703 (1964)
17 SMITH C H [illegible]
[illegible] Thalassemia I Pathogenesis and diagnosis Austr paediat J *4* 156 (1968)
20 WOLMAN, I J Transfusion therapy in Cooley's anemia Growth and health as related to long range haemoglobin levels A progress report Ann N Y Acad Sci *119* 736 (1964)

Authors' address Dr ISKANDAR WAHIDIJAT, Dr A H MARKUM and Dr Z. K ADANO, Division of Hematology and Child Surgery, Department of Child Health, Medical School, University of Indonesia, *Djakarta* (Indonesia)

After splenectomy, 14 children with thalassemia major (from group A) still needed blood transfusions, but with much less frequency. Before the spleen was removed, the children needed 3–4 series of blood transfusions in a year as against only 0–2 series after the operation. During the follow-up of 46–84 months post splenectomy, 3 of the 14 children no longer needed blood transfusion. Only 2 of the 25 group-A children expired, within 14- and 105 month follow-ups, respectively. One of them suffered from thalassemia Hb E disease. The cause of death in these 2 children was bronchopneumonia and purulent meningitis.

Patients with thalassemia-Hb E disease came usually at an older age to the doctor with complaints of distended abdomen and/or moderate anemia. They usually showed milder symptoms than those of thalassemia major. Most of these patients did not require anymore blood transfusions during the follow-up of 52.3 (14–186) months post splenectomy, although they were operated on at an older age (92.5 months). If we compare the results of the operations of the 14 thalassemic children from group A, who were operated on in very good condition, with those of the other 19 children from group B who were operated on after signs of hypersplenism and hemosiderosis appeared, it is evident that the first group of children had a more hopeful future than the latter. The most important problem in planning splenectomy in children with thalassemia is to decide the most proper moment to perform the operation. Various authors, such as Robinson and Sturgeon [15], did not find any correlation between the high incidence of infections following operation and the age of splenectomy. When it is feasible, the authors prefer to perform splenectomy on older children (more than 2 years of age), provided that there is no sign of hypersplenism or iron over-loading. But splenectomy should not be postponed if there are increased transfusion requirements, even when the child is below 2 years of age. Splenectomy done on healthy and older children who suffer from traumatic spleen rupture is seldom followed by overwhelming infections [7, 11, 13, 15]. Next to splenectomy, administration of desferrioxamin and high level packed cell transfusions [20] may prolong the life of children with thalassemia. However, some authors [3] do not see any benefit of high-level transfusions.

References

1 Betke, K., Marti, H. R., and Schlicht, I.: Estimation of small percentage of foetal hemoglobin. Nature, Lond. *184*: 1877 (1959).

2 BOURONCLE B A and DOAN C A Cooley s anemia indications for splenectomy Ann N Y Acad Sci *119* 709 (1964)
3 BROOK, C. G D THOMPSON E. N MARSHALL, W C and WHITEHOUSE, R. H Growth in children with thalassemia major and effect of two different transfusion regimens Arch Dis Childh *44* 612 (1969)
4 DACIE J V and LEWIS S M Practical hematology 3rd ed (Churchill London 1963)
5 ELLIS J T SCHULMAN I., and SMITH C. H Generalized siderosis with fibrosis of liver and pancreas in Cooley s (Mediterranean) anemia, with observations on pathogenesis of siderosis and fibrosis Amer J Path *30* 287 (1954)
6 ELLIS E F and SMITH R T The role of the spleen in immunity with special reference to the post splenectomy problem in infants Pediatrics *37* 111 (1966)
7 GOFSTEIN R and GELLIS S S Splenectomy in infancy and childhood. The question of overwhelming infection following operation Amer J Dis Child *91* 566 (1956)
8 GROSS L. Studies on the gross and minute anatomy of the spleen in health and disease J med Res *39* 311 (1919) taken from Ann Surg *136* 239 (1952)
9 HAGBERG B The iron binding capacity of serum in infants and children Acta paediat *42* suppl., vol 93 (1953)
10 HORAN M B and COLEBATCH J H Relation between splenectomy and subsequent infection Arch Dis Childh *37* 398 (1962)
11 HORAN M B and COLEBATCH J H Is splenectomy the cause of subsequent serious infections? Proc 9th Congr Int Soc Haemat 1962, vol 3 p 579 (1964)
12 ISKANDAR W MARKUM A H ADANG Z. K., and SULINDRO K Splenectomy on children with thalassemia. Paediat indones *9* 29 (1969)
13 MAC KENNON W M P BOLEY S J and MANFEL J Infection in children following splenectomy for traumatic rupture Amer J Dis Child *98* 710 (1959)
14 MOTULSKY A G CASSERD F GIBLET E. R BROWN G O., jr and FINCH C. A Anemia and the spleen New Engl J Med *259* 1164 (1958)
15 ROBINSON T W and STURGEON P Post splenectomy infection in infant and children Pediatrics *25* 491 (1960)
16 SCHORR J B and RADEL, E. Transfusion therapy and its complications in patients with Cooley s anemia Ann N Y Acad Sci *119* 703 (1964)
17 SMITH C H ERLANDSON M E SCHULMAN I., and STERN G Hazard of severe infections in splenectomized infants and children Amer J Med *22* 390 (1957)
18 SMITH C. H ERLANDSON M E STERN G and HILGARTNER M W Post splenectomy infection in Cooley s anemia New Engl J Med *266* 737 (1962)
19 TAFT L. E and MILLER H G Thalassemia I Pathogenesis and diagnosis. Austr paediat J *4* 156 (1968)
20 WOLMAN I J Transfusion therapy in Cooley s anemia Growth and health as related to long range haemoglobin levels. A progress report Ann N Y Acad Sci *119* 736 (1964)

Authors address Dr ISKANDAR WAHIDIJAT Dr A H MARKUM and Dr Z. K. ADANG Division of Hematology and Child Surgery Department of Child Health, Medical School University of Indonesia *Djakarta* (Indonesia)

times were within normal limits (11 and 65 sec, respectively) Random glucose con centration was 86 mg%

There were numerous megakaryocytes in the bone marrow aspirate, and the appearances were fully consistent with idiopathic thrombocytopenic purpura Erythropoiesis was normoblastic, granulopoiesis was normal and cellularity was within normal limits

The platelet count rose to 110,000 mm³ 10 days after the institution of prednisone therapy, but fell to 52,000 mm³ as the dose was gradually reduced and then discontinued However, purpura had disappeared and menorrhagia had ceased Prednisone was reinstituted after recurrence of bruising and bleeding, with the platelet count at 26 000 mm³, however, the spleen had to be removed eventually because large doses were needed to control symptoms The platelet count rose to 255,000/mm³ by the third post operative day and has remained above 200 000 mm³ since then, except for a temporary fall during a recent pregnancy

Three years previously, this patient had been treated for subacute glomerulonephritis with nitrogen mustard and corticosteroids (a total of 4 7 g of prednisone over 15 months) No haematological abnormality was noted during this period and her urine was subsequently free of albumin

Pathology The spleen weighed 140 g and was normal macroscopically Histologically, there were masses of large histiocytes in the splenic pulp, with foamy, slightly basophilic cytoplasm containing scattered fine granules (fig 1) These cells were stained with Sudan Black and Oil Red O, the granules were PAS positive Tissue fixed in formalin for about 11 days was then taken for electron microscopy, transferred to glutaraldehyde and osmium tetroxide in sequence, then embedded in epoxy resin Sections cut with an LKB microtome and stained with a combination of uranyl acetate and lead citrate were examined with a Phillips 100C electron microscope Sections 1 μm thick were stained with toluidine blue for light microscopy

Demonstrable in the histiocytes on electron microscopy were numerous 'myelin bodies' and occasional degenerate cells, some of which were without nuclei and contained dense bodies glycogen and small mitochondria (fig 2) Comparison with platelets free in the sinusoids suggested that these could be phagocytosed platelets The remainder of the splenic cells showed no remarkable features

Case 2

This 16-year-old Chinese female presented with a 3-month history of easy bruising and menorrhagia of 11 days duration On examination, she was anaemic and there were purpura and ecchymoses on her thighs and forearms The spleen was not enlarged and there was no lymphadenopathy She denied drug intake and exposure to chemicals. Hb, 6 8 g%, haematocrit 21%, white cell count, 5 200 mm³, with a normal differential count platelet count 12,000 mm³ LE cells and antinuclear factor were not demonstrable on several occasions Sternal marrow aspirate contained numerous megakaryocytes Erythropoiesis was normoblastic and leucopoiesis was normal

Idiopathic thrombocytopenic purpura was diagnosed and prednisone started immediately Within 3 days of her admission the bleeding *per vaginam* had decreased significantly, her platelet count rose to 205 000 mm³ on the seventh day The prednisone dosage was then gradually reduced and discontinued 5 weeks later



Fig 3 Case 2 A histiocyte containing degenerate particles, including one resembling a platelet. Uranyl acetate and lead citrate, × 27,600

Prednisone was restarted 3 months later when purpura and menorrhagia had recurred and the platelet count had fallen to 35,000 mm³ Her spleen was removed after 12 weeks of therapy The platelet count rose immediately after splenectomy and has remained above 200 000 mm³, although prednisone dosage was quickly reduced and discontinued within one month post operatively

Pathology The spleen weighed 70 g and was normal macroscopically Histologically, there was normal lymphoid tissue, but scattered in the pulp singly and in small groups were large pale histiocytes, similar to those of case 1, but much fewer in number Tissue from this spleen was taken for electron microscopy a few minutes after splenectomy, fixed in glutaraldehyde and osmium tetroxide and processed as for case 1

Histiocytes with myelin figures, in greater number than was expected from the light microscopic appearance, were seen on electron microscopy, some of them contained structures resembling degenerate platelets (fig 3)

Discussion

Splenic histiocytosis occurs in diabetes, thalassaemia [6], and the primary lipidoses, but our patients did not have any of the clinical features of these disorders We believe that they may be further examples of the

mation of such resting bone-marrow lymphocytes during regeneration of an aplastic bone marrow were shown [6, 7]

It was the purpose of this study to investigate the cytokinetic behaviour of bone marrow lymphocytes after primary and secondary immune stimulation, since bone marrow lymphocytes are involved in cellular events leading to antibody production [10–12] Specifically, the aim was to follow up that fraction of bone marrow lymphocytes which show a low turnover rate in adult life and to compare it to the slowly-turning-over lymphocytes in the spleen The retention of ^{3}H-TdR label could be a tool for detecting a transformation of cells after immune stimulation

Material and Methods

The experiments were performed in Lewis rats of either sex (FW 134 + 4 Wistar Institute) The animals were subdivided into 4 groups Two control groups (a and b) of 6 animals each and 2 experimental groups (A) of 20 animals and (B) of 18 animals

The animals of group a and A were injected subcutaneously every 8 h with 0.2 μCi of ^{3}H TdR[1]/g body weight through the first 6 weeks of life After another 4 weeks (i e., 10 weeks after birth) most of the slowly turning-over cells were still ^{3}H TdR labelled while nearly all cells rapidly proliferating by divisions had lost their labelling beyond their autoradiographic detectability

Primary immune response experiment At this time the animals of group A received 500 μg human globulin intravenously mixed with complete Freund s adjuvant Two animals each were sacrificed 1 2 3 4 5 6 8 11, 14 and 18 days respectively, after administration of antigen Group a which received no antigen served as control Three animals of group a were killed when 10 weeks old the other three 8 days later

At an age of 5 weeks the animals of group B and b received 500 μg human γ globulin mixed with complete Freund s adjuvant subcutaneously divided into 3 portions (right fore and hindfoot pad and thoracic wall) Immediately afterwards these rats received subcutaneously every 8 h 0 2 μCi of ^{3}H TdR/g body weight for one week After discontinuation of ^{3}H TdR injections no other treatment was given for 4 weeks

Secondary immune response experiment At this time when the animals of group B were 10 weeks old they again received the same antigen in the mode mentioned above Two animals each were sacrificed 1 1 5 2 3 4 5 6 8 and 11 days after the second administration of antigen Group b which received no secondary immune stimulus served as control Three animals of this group were killed when 10 weeks old the other three 8 days later

[1] ^{3}H TdR (specific activity 1 9 Ci mM) was obtained from the Radiochemical Centre Amersham UK

Processing of Materials

Antibody titre The titre of anti HGG in the blood was measured using the passive haemagglutinat on technique of STAVITSKY [13] modified by the micro-method of TAKATSY [14]

Bone marrow The right femur was used for bone marrow smears The bone marrow of the left femur between the epiphyses was blown out and then suspended in 5 ml of a solution of 1 part 1 10⁴° • Na_2 FDTA in 0 7° • saline and 4 parts of 0 9% saline From this suspension 0 02 ml was diluted in 10 ml of Isoton®* with 0 1 ml of Zaponin®* The concentration of nucleated cells was then determined in a haemocytometer and the number of nucleated cells mg bone marrow calculated Autorad ographs of bone marrow smears were prepared using Kodak AR 10 stripping film exposed for 61 days and stained with Giemsa solution at pH 5 75

In the smears the number of labelled cells per 1 000 nucleated bone marrow cells was counted and d fferentiated into 4 morphologically d stinct cell classes The mean grain count/labelled cell was determined from 50 labelled cells of each cell class

Autoradiographs of unlabelled smears showed that the background was less than 4 grains nucleus Labelled cells were therefore considered to be cells with 4 or more grains

Spleen After determination of the spleen weight, the organ was fixed in Carnoy s fixative Histologic slices cut at 4 μm were autoradiographed using Ilford L4 Emulsion with an exposure time of 33 days and stained with haematoxylin eosin and methylgreen pyronin In the histoautoradiographs the labelling index and the labelling intensity were determined over 500 lymphocytes, 100 reticulum cells of the right pulp and 100 cells of the histiocytic area around the right pulp The background was negligible hence no correction was necessary

Results

Antibody

During the primary immune response after intravenous administration of HGG (group A) antibodies were first detected on day 4 Maximum levels were reached on day 6 with a titre of 1 160, followed by a fast decrease of antibody concentration in the blood During the secondary immune response after subcutaneous administration of HGG (group B) antibody titre began to rise on day 2 or 3 Maximum titres of 1 5,120 were found from day 4 to day 8, with a slight decrease on day 11

Behaviour of Resting Cells in the Primary Response Experiment

Bone marrow Before the application of antigen, when the animals were 10 weeks old, 4 different types of bone marrow cells were still la

* Coulter Electronics Ltd, UK.

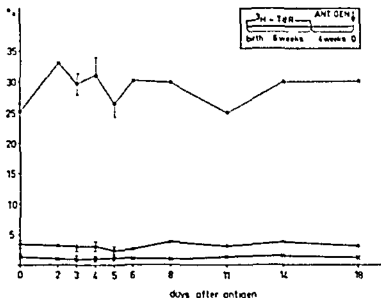


Fig 1 Amount of unlabelled lymphocytes and number of labelled matrix cells, and labelled lymphocytes i e , cytokinetically, 'resting' cell populations of the bone marrow as percentage of total bone marrow cells at different times after primary immune stimulation (vertical bars indicate the SEM) ● Unlabelled lymphocytes, ○ labelled matrix cells, × labelled lymphocytes

belled These cells were classified morphologically as lymphocytes and 3 types of matrix cells reticular cells type A, reticular cells type B and endothelial cells The morphological classification of these cells is given elsewhere [5] The total number of nucleated cells/mg bone marrow was $1\ 78 \pm 0\ 08 \times 10^6$ (SEM) The number of labelled matrix cells amounted to $0\ 049 \pm 0\ 002 \times 10^6$, the number of labelled lymphocytes, $0{,}027 \pm 0\ 0005 \times 10^6$/mg bone marrow

Since only 6 6% of all bone marrow lymphocytes was labelled, the number of unlabelled lymphocytes was calculated to be $0\ 38 \pm 0\ 006 \times 10^6$/mg bone marrow The mean grain count was 17 for lymphocytes, 28 for endothelial cells, 23 for reticular cells A, and 24 for reticular cells B

The absolute number of bone-marrow cells after primary immune stimulation showed only uncharacteristic oscillations As indicated in figure 1, the amount of labelled matrix cells, labelled lymphocytes and unlabelled lymphocytes at different times after primary immune stimulation was found to be constant Since no variation in the distribution and the percentage of labelled endothelial and reticular cells could be found, these

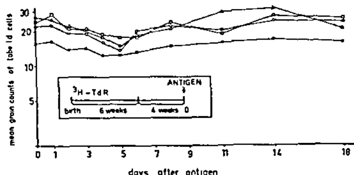


Fig 2 Labelling intensity of slowly renewing bone marrow cells as a function of time after primary immune stimulation △ Endothelial cells □ reticular cells B, ○ reticular cells A, × lymphocytes

cells are summarised in the figure as matrix cells At no time was any new cell class found to be labelled Thus, there was no sign of transformation from one labelled cell line into another The labelling intensity of slowly-turning-over bone-marrow cells after primary immune stimulation is shown in figure 2 There was obviously no change of labelling intensity over lymphocytes or matrix cells during the whole time of observation

Spleen Spleen weight was 392 g before immunisation, rose to 455 g one day after i v antigen injection and reached its maximum with 651 g after 18 days This was accompanied by a hyperplasia of the white pulp, reaching its maximum after 8 days

The labelling index of small lymphocytes, which was 50 3% before antigen injection, remained unchanged until day 3 and then dropped gradually down to 35 5% after 18 days, while the labelling intensity remained nearly constant The labelling index of reticular cells decreased in the first 3 days after antigen injection from 67 8 to 55 9% and remained constant thereafter up to day 18 The mean grain count of reticular cells remained unchanged The labelling index of histiocytes decreased after antigen application from 32 9 to 21 9% on day 18, accompanied by a minimal diminution of mean grain count No wave of labelled pyroninophilic blast cells in the white pulp could be observed However, a varying fraction of blast cells (mostly between 10 and 20%) was found to be labelled

In the spleens of the control animals (group a), some alteration of the labelling pattern was observed Eight days after the experimental rats (group A) had received antigen, the labelling indices of the small lympho-

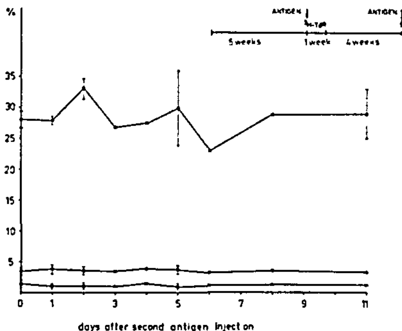


Fig 3 Amount of unlabelled lymphocytes and number of labelled matrix cells, and labelled lymphocytes, i e , cytokinetically, 'resting cells of the bone marrow as percentage of the total bone marrow cells at different times after secondary immune stimulation vertical bars indicate the SEM) ● unlabelled lymphocytes ○ labelled matrix cells, × labelled lymphocytes

cytes and histiocytes were slightly reduced to 45 9 (as compared to 41 5% in the experimental rats) and to 27 7% (as compared to 22 0%) respectively, whereas the labelling index of the reticular cells and of the blast cells remained nearly constant The mean grain count of the different cell types and the spleen weight in the control animals remained unchanged

Behaviour of Resting Cells in the Secondary Response Experiment

Bone marrow Before the beginning of the second application of antigen *4 weeks after ³H-TdR, again only 4 cell types were still labelled* Morphologically, these cell classes were similar to those described above The total number of nucleated cells/mg bone marrow was 1 77 ± 0 1 × 10^4, the number of labelled matrix cells, 0 059 ± 0 003 × 10^4, the number of labelled lymphocytes, 0 019 ± 0 0005 × 10^4 (Corresponding to a labelling index of bone-marrow lymphocytes of 3 9%), the number of unlabelled lymphocytes was 0 49 ± 0 008 × 10^4 The mean grain count was 12 for lymphocytes, 25 for endothelial cells, 19 for reticular cells, type A and 23 for reticular cells type B

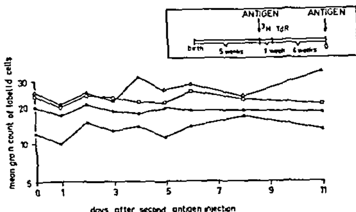


Fig 4 Labelling intensity of slowly renewing bone marrow cells as a function of time after secondary immune stimulation. △ Endothelial cells □ reticular cells B ○ reticular cells A, × lymphocytes

As indicated in figure 3, no change in the number of labelled endothelial cells and reticular cells, summarised as matrix cells, could again be shown after secondary immune stimulation The percentage of labelled lymphocytes also remained constant during the whole time of observation On the other hand, there was no demonstrable variation of cell numbers for unlabelled lymphocytes and other nucleated bone marrow cells There was no appearance of labelled blast cells, as should be expected if a measurable quantity of labelled cells had been transformed An increase in plasma cell or primitive blast cell number was also not observed The mean grain count as a function of time of slowly-turning-over bone-marrow cells after secondary immune stimulation, as indicated in figure 4, showed no change in the labelling intensity of all cell classes

Spleen The spleen weight was 320 mg before the second subcutaneous antigen injection and increased to 400–500 mg from day 1 to day 8 after antigen The weight increase of the spleen was not accompanied by such remarkable histologic changes of the organ as was the case after the i v antigen injection during the primary response The labelling index of small lymphocytes decreased during the secondary response from 44 4 before antigen to 35 0% after 11 days, while labelling intensity remained nearly constant

The labelling index of reticular cells decreased minimally from 56 7 to 49 2% on day 11, the labelling intensity remaining constant The histo-

cytes showed a decrease of their labelling index from 33.5 before antigen to 10.0% 11 days after the second antigen injection, without changing their labelling intensity. There was a slight decrease of labelling index of the blast cell population, from 15.8 before antigen to 9.5% 11 days after antigen.

In the spleens of the control animals (group b), the labelling indices of the small lymphocytes and histiocytes were reduced slightly to 39.8% (as compared to 35.8% in the experimental animals) and to 28.5% (as compared to 21.5% in the experimental animals), respectively, whereas the labelling index of the reticular cells remained constant. The mean grain count of the different cell types remained unchanged. The spleen weight also did not change during the observation time.

Discussion

In earlier experiments of our group, a fraction of slowly-turning-over lymphocytes in bone marrow could be found in the order of 5%, this fraction was shown to be labelled for weeks and months afterwards [5]. Since the development of immuno-competent cells is a postnatal phenomenon, it was felt that the method applied could serve for the investigation of an immune stimulation of slowly-turning-over cells.

Specifically, the question remains which cell type could serve as a precursor cell pool of immunological reactive cells and whether these precursor cells are, cytokinetically, resting or proliferating cells. The small fraction of labelled long living bone-marrow lymphocytes apparently did not react with an increased turnover to primary and secondary immune stimulation with HGG + CFA. This can be due to 2 reasons. (1) The bone-marrow lymphocytes that react to antigens [10, 12] belong to the large class of short-living (in our study, naturally non-labelled) bone marrow lymphocytes [3, 4]. (2) It is, nevertheless, theoretically possible that our results are falsely negative in the sense that some labelled bone-marrow lymphocytes are also engaged in the immune response to HGG, but that the method of autoradiography is not sensitive enough to detect the loss of very few labelled cells. In the spleen of mice only 0.10–0.20 immunocompetent cells for a specific antigen could be detected per 10^4 cells [1, 2, 8]. Therefore, the conclusion would be that under immune stimulation, the mass of long-living lymphocytes does not react in the sense of a transformation or proliferation. In contrast to that behaviour, as could be

shown in previous work, the mass of bone-marrow lymphocytes did react under regeneratory stimulus [6, 7].

The turnover of long living spleen lymphocytes was accelerated by antigen – a striking difference from the findings in bone marrow. The decrease of labelling index (marked decrease after application of antigen and moderate decrease under the influence of continuous stimulation by background antigen) may be caused by 2 factors: (1) Transformation of labelled lymphocytes into immunoblasts, as is indicated by the findings in stimulated lymph nodes in the experimental series [15], and (2) formation of new unlabelled lymphocytes during the immune response [9, 17].

The matrix cells of the bone marrow did not change their labelling pattern after exposure to an antigen. Therefore, the interpretation would again be that only few, if any, of these cells did react to antigen stimulation. Similar findings could be demonstrated under regeneratory demand [6, 7]. However, the reticular cells and histiocytes of the spleen showed a decrease of their labelling index without a marked diminuation of the mean grain count. Our best possible explanation for this fact would be that new unlabelled cells from the reticulo-endothelial system have migrated into the enlarged spleen after antigen application.

References

1 Albright, J. F. and Makinodan, T.: Dynamics of expression of competence of antibody producing cells; in Sterzl: Molecular and cellular basis of antibody formation, pp. 427–446 (Academic Press, New York 1965).

2 Armstrong, W. D. and Diener, E.: A new method for the enumeration of antigen reactive cells responsive to a purified protein antigen. J. exp. Med. *129*: 371–391 (1969).

3 Craddock, C. G.: Bone marrow lymphocytes of the rat as studied by autoradiography. Acta haemat., Basel *33*: 19–27 (1965).

4 Everett, N. B. and Caffrey, R. W.: Radioautographic studies of bone marrow small lymphocytes; in Yoffey: The lymphocyte in immunology and haemopoiesis, pp. 108–119 (Arnold, London 1967).

5 Haas, R. J.; Bohne, F., and Fliedner, T. M.: On the development of slowly t[illegible]

6 [illegible]

[illegible] 4: 31–45 (1971).

7 Bohne, F.; Haas, R. J.; Fliedner, T. M., and Fache, I.: The role of slowly proliferating cells in rat bone marrow during regeneration following hydroxyurea. Brit. J. Haemat. *19*: 533 (1970).

cytes showed a decrease of their labelling index from 33.5 before antigen to 10.0% 11 days after the second antigen injection, without changing their labelling intensity. There was a slight decrease of labelling index of the blast cell population, from 15.8 before antigen to 9.5% 11 days after antigen.

In the spleens of the control animals (group b), the labelling indices of the small lymphocytes and histiocytes were reduced slightly to 39.8% (as compared to 35.8% in the experimental animals) and to 28.5% (as compared to 21.5% in the experimental animals), respectively, whereas the labelling index of the reticular cells remained constant. The mean grain count of the different cell types remained unchanged. The spleen weight also did not change during the observation time.

Discussion

In earlier experiments of our group, a fraction of slowly-turning-over lymphocytes in bone marrow could be found in the order of 5%, this fraction was shown to be labelled for weeks and months afterwards [5]. Since the development of immuno-competent cells is a postnatal phenomenon, it was felt that the method applied could serve for the investigation of an immune stimulation of slowly-turning-over cells.

Specifically, the question remains which cell type could serve as a precursor cell pool of immunological reactive cells and whether these precursor cells are, cytokinetically, resting or proliferating cells. The small fraction of labelled long-living bone-marrow lymphocytes apparently did not react with an increased turnover to primary and secondary immune stimulation with HGG + CFA. This can be due to 2 reasons: (1) The bone-marrow lymphocytes that react to antigens [10, 12] belong to the large class of short-living (in our study, naturally non-labelled) bone-marrow lymphocytes [3, 4]. (2) It is, nevertheless, theoretically possible that our results are falsely negative in the sense that some labelled bone-marrow lymphocytes are also engaged in the immune response to HGG, but that the method of autoradiography is not sensitive enough to detect the loss of very few labelled cells. In the spleen of mice only 0.10–0.20 immuno-competent cells for a specific antigen could be detected per 10^4 cells [1, 2, 8]. Therefore, the conclusion would be that under immune stimulation, the mass of long-living lymphocytes does not react in the sense of a transformation or proliferation. In contrast to that behaviour, as could be

shown in previous work, the mass of bone marrow lymphocytes did react under regeneratory stimulus [6, 7]

The turnover of long living spleen lymphocytes was accelerated by antigen – a striking difference from the findings in bone marrow The decrease of labelling index (marked decrease after application of antigen and moderate decrease under the influence of continuous stimulation by background antigen) may be caused by 2 factors (1) Transformation of labelled lymphocytes into immunoblasts, as is indicated by the findings in stimulated lymph nodes in the experimental series [15] and (2) formation of new unlabelled lymphocytes during the immune response [9, 17]

The matrix cells of the bone marrow did not change their labelling pattern after exposure to an antigen Therefore, the interpretation would again be that only few, if any, of these cells did react to antigen stimulation Similar findings could be demonstrated under regeneratory demand [6, 7] However, the reticular cells and histiocytes of the spleen showed a decrease of their labelling index without a marked diminuation of the mean grain count Our best possible explanation for this fact would be that new unlabelled cells from the reticulo endothelial system have migrated into the enlarged spleen after antigen application

References

1 Albright J F and Makinodan T Dynamics of expression of competence of antibody producing cells in Sterzl Molecular and cellular basis of antibody formation pp 427–446 (Academic Press New York 1965)

2 Armstrong W D and Diener E A new method for the enumeration of antigen reactive cells responsive to a purified protein antigen J *exp Med 129* 371 391 (1969)

3 Craddock, C. G Bone marrow lymphocytes of the rat as studied by autoradiography Acta haemat., Basel *33* 19–27 (1965)

4 Everett N B and Caffrey, R W Radioautographic studies of bone marrow small lymphocytes in Yoffey The lymphocyte in *immunology and haemopoiesis*, pp 108–119 (Arnold London 1967)

5 Haas, R J Bohne F., and Fliedner T M On the development of slowly turning over cell types in neonatal rat bone marrow Blood *34* 791–805 (1969)

6 Haas R J Bohne F., and Harriss E B Cytokinetic analysis of *slowly proliferating* bone marrow cells *during recovery from* radiation injury Cell Tissue Kin 4 31–45 (1971)

7 Bohne, F Haas R J, Fliedner T M, and Fache, I The role of slowly proliferating cells *in* rat bone marrow during regeneration following hydroxyurea Brit J Haemat *19* 533 (1970)

8 KENNEDY, J C., TILL, J E., SIMINOVITCH L., and MCCULLOCH, E A The proliferative capacity of antigen sensitive precursors of hemolytic plaqueforming cells J Immunol *96* 973–980 (1966)

9 MCGREGOR, D D, KOSTER, F T., and MACKANESS, G B The short living small lymphocytes as a mediator of cellular immunity Nature Lond *228* 855–856 (1970)

10 MITCHELL, G F and MILLER J F A P Cell to cell interaction in the immune response II The source of hemolysin forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J exp Med *125* 821–837 (1968)

11 RABELLINO F, COLON, S, GREY, H M., and UNANUE, E. R Immunoglobulins on the surface of lymphocytes I Distribution and quantitation J exp Med. *133* 156–167 (1971)

12 SINGHAL, S K and RICHTER, M Cells involved in the immune response IV The response of normal and immune rabbit bone marrow and lymphoid tissue lymphocytes to antigens *in vitro* J exp Med *128* 1099–1128 (1968)

13 STAVITSKY, A B Micromethods for the study of proteins and antibodies. I Procedure and general applications of hemagglutination and hemagglutination inhibition reactions with tannic acid and protein treated red blood cells J Immunol *72* 360–367 (1954)

14 TAKATSY, G The use of spiral loops in serological and virological micro methods Acta microbiol hung *3* 191–202 (1955)

15 TREPEL, F and FRÖHLICH D Unpublished data

16 WALB D, HAAS R J und TREPEL, F Autoradiographische Untersuchungen zum Verhalten der Lymphozyten im peripheren Blut nach xenogener Hauttransplantion bei Ratten Blut *23* 84–91 (1971)

Author s address Priv Doz Dr med R J HAAS Abteilung für Kinderheilkunde der Universitat Ulm Prittwitzstrasse 43 *79–Ulm Donau* (FRG)

Régénération de la moelle osseuse irradiée après l'autotransplantation de la moelle protégée

Acta Haemat 48 49–57 (1972)

Régénération de la moelle osseuse irradiée après l'autotransplantation de la moelle protégée

VUKOSAVA NINKOV, OLGA PILETIĆ NEVENKA STOJANOVIĆ, DUŠANKA STEPANOVIĆ et M BRADIĆ

Institut des Sciences Nucleaires «Boris Kidrič» Vinča,
Institut d'Histologie de la Faculté de Medicine Belgrade

Abstract Regeneration of bone marrow cells was studied in irradiated rats following autotransplantation of protected bone marrow *in situ* into the circulation immediately after irradiation. Animals undergoing bone marrow autotransplantation show much more intense regenerat on those with bone marrow *in situ* probably because of the different rates of transportation of the mobile strain cells to the haemopoietic organs and their number and speed of introduction into the circulation

Key Words
Bone marrow autotransplantation
Bone marrow regeneration
Irradiation of bone marrow
Rat bone marrow

La theorie sur la recolonisation de la moelle osseuse et de la rate apres irradiation au moyen des cellules-souche mobiles introduites par la transplantation ou endocolonisees d une region hematopoïetique non irradiee dans une région hématopoietique irradiee a été confirmee dans une serie de travaux [1, 3, 5, (

point de vue theorique, si la

tion d un tissu hematopoïétiq

maux est effective à differents degres Il a éte constate que la depopulation des cellules-souche et l'endocolonisation de la moelle osseuse et de la rate après irradiation sont bien plus intenses chez la souris que chez d autres animaux (rats, singes) et chez l homme [13]

L'effet positif enregistre dans les travaux précedents traitant la dynamique de l effet mutuel de la moelle osseuse protégee et irradiee, ainsi que les processus de la regéneration apres irradiation en présence de la moelle osseuse protegee *in situ* dans l'organisme irradie a ete insuffisant

[11] Les questions qui se posent sont les suivantes combien et en quel nombre les cellules-souche des rats sont-elles mobiles, et puis les cellules-souche mobiles seront-elles reçues par l'organe récepteur?

Dans ce travail nous avons examiné aussi la dynamique de la régénération hématopoïétique et la réactivité des cellules souche de la moelle osseuse chez les rats dont la moelle osseuse protégée a été restée *in situ* et chez lesquels elle a été autotransplantée dans la circulation immédiatement après l'irradiation

Matériel et Méthodes

Les expériences ont été faites avec des rats blancs femelles souche Wistar, poids 160–180 g Les animaux ont été irradiés par la dose de 800 r, intégralement, sauf une *extrémité* arrière qui a été protégée Les animaux ont été narcotisés avant l'irradiation par «Nembutale» (le sodium de pentobarbital) Une patte arrière a été protégée avec du plomb en forme cylindrique de 5 mm d'épaisseur

Les conditions de l'irradiation 16 mA, 200 kV, distance 42 cm vitesse de dose *115 r/min, dose donnée 800 r*

La recolonisation de la moelle osseuse après irradiation a été observée du 1er au 10e jour par des paramètres suivants cellularité générale de la moelle osseuse de tibia nombre général des cellules érythroïdes dans le tibia, activité proliférique de cette population [4]

Le nombre de réticulocytes et d'érythrocytes dans le sang périphérique a été déterminé par les méthodes de routine

L'autotransplantation de la moelle osseuse à partir de l'extrémité protégée a été faite immédiatement après irradiation Une suspension de cellules prélevées dans la moelle osseuse de tibia est mise dans la solution de Tyrode et injectée dans la veine de queue Le nombre de cellules données a été de $1\text{-}2 \times 10^7$

Résultats

La cellularité générale de tibia pendant la période d'observation du 1er au 10e jour après irradiation montre que la recolonisation de la moelle osseuse chez les animaux autotransplantés de la moelle protégée est incomparablement plus intense que celle observée chez le groupe ou la moelle osseuse est restée *in situ* La repopulation spontanée dans la moelle osseuse chez les témoins retarde de 48 h, elle est présentée par un petit nombre de cellules (tab I, fig 1)

Les valeurs des cellules érythroïdes sont tout à fait en accord avec celles de la cellularité générale La différence du comportement chez le

Tableau I Cellularité générale des cellules nucléées de tibia (× 10³)

Traitement	Jours après traitement				
	1	3	5	7	10
800 r. contrôles	7 456 ± 1 812 (6)	1 125 ± 304 (5)	1 285 ± 301 (5)	2 627 ± 1 042 (6)	3 700 ± 896 (6)
800 r + moelle osseuse protégée *in situ*	8 190 ± 1 880 (5)	3 088 ± 1 149 (4)	3 240 ± 419 (5)	4 709 ± 1 802 (4)	13 475 ± 4 239 (4)
800 r + autotransplant de moelle osseuse protégée	7 407 ± 1 375 (6)	2 382 ± 706 (14)	5 211 ± 1 825 (14)	13 914 ± 2 531 (15)	21 890 ± 3 450 (5)

Valeur normale 15 740 ± 374 (5)
Entre parenthèses le nombre d animaux

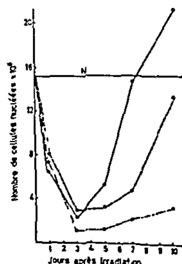


Fig 1 Cellularité générale des cellules nucléées de tibia N = Valeurs normales, •— —• Témoin irradié par 800 r •———• Animal irradié par 800 r avec de la moelle osseuse protégée *in situ* •——• Animal irradié par 800 r après autotransplantation de la moelle osseuse protégée

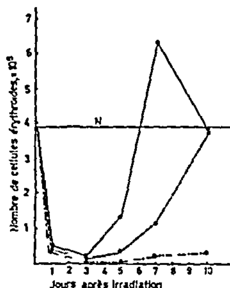


Fig 2 Valeurs absolues des cellules érythroïdes nucléées de tibia Légende comme dans la figure 1

Tableau II Nombre de cellules érythroïdes par rapport à 1000 autres cellules nucléées (Er/Nc)

Traitement	Jours après traitement				
	1	3	5	7	10
800 r, contrôles	53/947±30	–	26/974±19	71/929±73	30/970±73
800 r + moelle osseuse protégée *in situ*	45/955±7	41/959±54	104/896±25	247/753±99	282/718±50
800 r + autotransplant. de moelle osseuse protégée	55/945±13	46/954±13	262/738±96	425/575±90	305/695±54
Valeur normale	250/750±59				

groupe ayant subi l'autotransplantation est constatée le 10e jour au moment où la cellularité érythroïde diminue et la cellularité générale des cellules nucléés dans la moelle osseuse augmente La différence apparaît parce qu'entre le 7e et le 10e jour commence la myélopoïèse

Tableau III Index statmocinétique des cellules érythroïdes de la moelle osseuse (0 00)

Traitement	Jours après traitement				
	1	3	5	7	10
800 r	–	–	–	109±8	162±17
contrôles	(5)	(5)	(5)	(5)	(5)
800 r + moelle	–	–	83±16	141±37	179±23
osseuse protégée *in situ*	(5)	(5)	(5)	(4)	(4)
800 r + autotransplant de moelle	–	–	350±89	366±93	338±51
osseuse protégée	(3)	(3)	(5)	(5)	(6)

Valeur normale 198±38 (8)
Entre parenthèses le nombre d animaux

intense laquelle repousse l hyperprolifération des cellules erythroïdes et les rapports entre les deux souches se normalisent progressivement (fig 2) Les valeurs du rapport relatif des cellules erythroïdes calculées à 1000 cellules nucléees dans la moelle osseuse sont presentées dans le tableau II

L'activité mitotique des cellules erythroïdes a eté suivie par les index statmocinétiques (tab III, fig 3) Une activité mitotique négligeable est constatée chez le groupe témoin dans la population érythroide de la moelle osseuse seulement le 7e jour après irradiation En présence de la moelle osseuse protegee *in situ*, la prolifération des cellules érythroides commence chez les animaux déjà le 5e jour apres irradiation elle atteint 50% de la valeur normale Une hyperprolifération intense des cellules éryhtroïdes commence simultanément, elle est 175% de la valeur normale Les différences sont très grandes du point de vue statistique (p<0 001, *P* = *5,9*)

Les réticulocytes périphériques sont calcules en valeurs absolues par rapport au nombre d érythrocytes dans 1 mm³ de sang La premiere augmentation après l irradiation est constatée le 5e jour déjà 23 088 à 1 mm³ chez les animaux en presence de la moelle osseuse protegee *in situ* 58 007 à 1 mm³ chez les animaux autotransplantes L'apparition des réticulocytes periphériques n a pas du tout été decelée Elle n a pas été constatée même le 7e jour de l observation dans le sang périphenque chez les groupes temoins

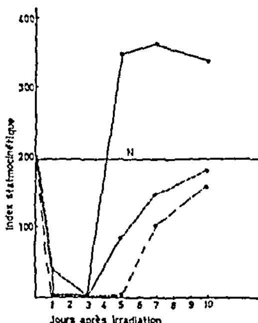


Fig 3 Index statmocinétique des cellules érythroïdes de la moelle osseuse Légende comme dans la figure 1

Des analyses quantitatives et histologiques de la rate, présentées dans un prochain rapport [12], prouvent les résultats obtenus par d'autres méthodes et précisent le cours de l'endocolonisation et de la différenciation hématopoïétique dans différentes conditions expérimentales

Discussion

Après une série de faits expérimentalement prouvés on a tiré la conclusion que la régénération du tissu hématopoïétique est due à la cellule-souche Les processus régénératifs spontanés commencent plus tôt ou plus tard en fonction de la dose d irradiation donnée et de la différence en radiosensibilité et en temps nécessaire à la réparation des cellules souche chez différentes espèces d'animaux expérimentaux [8, 11]

Après une série d'expériences avec la protection de certaines régions des tissus hématopoïétiques ou le traitement de transfusion des cellules normales de la moelle osseuse on a tiré les conclusions suivantes la cellule-souche est une cellule mobile capable de déménager d un tissu hématopoïétique non irradié dans le tissu hématopoïétique irradié ou de

le repeupler après la transplantation, puis de continuer à se diviser et à se différencier dans n'importe quelle cellule hématopoïétique du tissu ou de l'organe récepteur.

L'établissement des processus hématopoïétiques après l'irradiation ainsi que la transplantation de la moelle osseuse normale est très rapide. La vitesse d'établissement des processus régénératifs après l'irradiation dépendra du nombre des cellules souche introduites par la transplantation [2]. Les cellules de la poule prolifératique introduites hématopoïétiquement nettement différenciées quittent la moelle osseuse du récipient après maturation et ne contribuent pas à la régénération de la moelle osseuse aplastique des récipients irradiés. La cellule-souche est la cellule autoreproductive et capable de se différencier dans n'importe quelle cellule hématopoïétique. Par conséquent, elle est responsable de la régénération du processus de l'hématopoïèse dans la phase après l'irradiation [7].

Outre la différence en radiosensibilité des cellules-souche il y a une différence en mobilité des cellules souche chez différentes espèces d'animaux. Strelin [13] indique les différences qui existent en vitesse d'implantation et en temps de population de pénétration spontanée dans la circulation des cellules-souche mobiles chez la souris, le rat, le singe et l'homme. Hellman *et al.* [6] indiquent les différences en sensibilité chez la poule des cellules-souche de la même espèce. Les cellules-souche de la poule sont remarquablement plus sensibles si on mesure leur capacité de donner des précurseurs érythroïdes.

Lamerton *et al.* [7] ont examiné la recolonisation de la moelle osseuse et l'établissement de l'hématopoïèse extramédullaire dans la rate chez les rats irradiés par 200 r en présence de la moelle osseuse protégée dans l'organisme. On a trouvé une différence en processus spontanés et stimulés de la recolonisation en ce qui concerne le temps et l'intensité en présence de la moelle osseuse protégée par rapport aux animaux témoins totalement irradiés. Dans un de nos travaux antérieurs nous avons examiné l'effet de la moelle osseuse protégée et irradiée chez les rats (les rats irradiés par 800 r, avec une extrémité arrière localement protégée). Nous avons prouvé qu'il y a un effet favorable de la présence de la moelle osseuse protégée *in situ* sur les processus de la régénération après irradiation dans la moelle osseuse sur la survie et le rétablissement par rapport aux animaux témoins totalement irradiés [11].

De Gowin [3] a étudié la prolifération et la différenciation des cellules de la moelle osseuse chez les souris dont une extrémité a été protégée lors

de l'exposition à des fortes doses Les résultats ont confirmé la migration intense des cellules souche mobiles et la repopulation de la moelle osseuse et en particulier celle de la rate chez les souris dont la moelle osseuse a été protégée *in situ* D'après DE GOWIN les cellules souche mobiles ont implanté, repliqué et formé des colonies de nouvelles cellules hématopoïetiques Après la réplique, les cellules souche commencent à répondre au stimulus de différenciation et les cellules spécialisées apparaissent dans le sang périphérique

Les résultats de FIHOKA *et al* [5] ont confirmé qu'il y a une grande différence entre la repopulation de la moelle osseuse et de la rate, après irradiation, chez les rats dont la moelle osseuse est protégée *in situ*, et que les contrôles ont été totalement irradiés

L'implantation ralentie, c'est-à-dire l'introduction spontanée d'un tissu moins irradié ou protegé hématopoïetiquement dans la circulation d'un nombre relativement plus petit des cellules souche mobiles représente la cause d'une recolonisation plus ralentie de la moelle osseuse ou de la rate chez les rats Il faudrait tenir compte du fait que l'organe récepteur chez les rats a un comportement passif à l'égard de la repopulation de la cellule souche ou qu'il y a une inhibition jusqu'à un temps déterminé, ce qui fait retarder la réponse hématopoïétique

Les résultats donnés dans ce rapport ont montre que la recolonisation est bien plus intense chez les animaux autotransplantés que chez les animaux ou la moelle osseuse est restée *in situ* Tout cela suggère un phénomène provoquant les processus de repopulation spontanée et ralentie On peut supposer que le nombre de cellules souche est petit, qu'elles sont transportées ou introduites dans la circulation ou dans d'autres organes récepteurs Les processus simultanés de la régénération dans les deux groupes expérimentaux sont très intenses par rapport aux témoins ce qui élimine la possibilité de recevoir les cellules-souche dépeuplées dans l'organe récepteur Il semble que le seul facteur décisif est un petit nombre de cellules souche repeuplant lors de la recolonisation spontanée en raison du transport difficile ou de l'introduction dans la circulation chez les rats

Résumé

Chez des rats irradiés la régénération des cellules de la moelle osseuse est examinée après l'autotransplantation de la moelle osseuse protégée *in situ* dans la circulation aussitôt après l'irradiation Les animaux ayant subi l'autotransplantation de la moelle osseuse présente une régénération de la moelle osseuse bien plus

intense que ceux en présence de la moelle osseuse *in situ*, ce qui est probablement dû aux différentes vitesses de transport des cellules-souche mobiles jusqu'aux organes hématopoïétiques, ainsi qu'au nombre et à la vitesse de leur introduction dans la circulation.

Bibliographie

1 BRECHER, A J, MCCULLOCH, E. A., and TILL, J E. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells Nature, Lond *117* 452–454 (1963)

2 CHERTKOV, J L Current views on histogenesis of hemopoietic tissue under conditions of transplantation Problemy gematologii i perelivannia krovy *5* 14–21 (1969)

3 DE GOWIN, R Erythroid differentiation during stem cell proliferation J Lab clin Med *70/1* 23–35 (1967)

4 DUSTIN, P The quantitative estimation of mitotic growth in the bone marrow of the rat by statmokinetic (colchicine) method, in STOHLMAN Kinetics of cellular proliferation, p 50 (Grune & Stratton, New York 1959)

5 FUJIOKA, S, HIROSHIMA, K., KUMATORI, T, TAKAKU, F., and NAKAKO, K.. Mechanism of hemopoietic recovery in the x irradiated mouse with spleen or one leg shielded Radiat. Res *31* 826–839 (1967)

6 HELLMAN, S., GRATE, H E., and CHAFFEY, J T Effects of radiation on the capacity of the stem cell compartement to differentiate into granulocytic and erythrocytic progeny Blood *34* 141–157 (1969)

7 LAMERTON L. T, BELCHER, E H, and HARRISS, E B Blood uptake of Fe^{59} in studies of red cell production, in STOHLMAN Kinetics of cellular proliferation, p 301 (Grune & Stratton, New York 1959)

8 LUDWIG, F C. and KOHN, H J Quantitative studies on the radiation patology of the bone marrow of small laboratory animals Marrow response to whole body x irradiation in rodents of different LD_{50} Radiat. Res *21* 233–243 (1964)

9 MARSH, J C., BOGGS, D R, BISHOP, C. R., CHERVENICK, P A, CARTWRIGHT, G E, and WINTROBE, M M Factors influencing hematopoietic spleen colony forming formation in irradiated mice I. The normal pattern of endogenous colony formation J exp Med *126* 833–849 (1967)

10 MCCULLOCH, E. A. and TILL, J E Proliferation of hemopoietic colony forming cells transplanted into irradiated mice Radiat. Res. *22* 383–397 (1964)

11 NINKOV, V Response of bone marrow cells after whole body irradiation and partial protection in rats. Strahlentherapie *134* 463–469 (1967)

12 NINKOV, V, PILETIĆ, O et STOJANOVIĆ, N (Sous presse

13 STRELIN, G S Regeneration after irradiation and bone marrow transplantation. Radiobiologia A.N SSSR *2* 31–33 (1968)

Adresse des auteurs Dr V NINKOV, Dr O PILETIĆ, Dr N STOJANOVIĆ, D STEPANOVIĆ et M BRADIĆ, Institut des Sciences Nucléaires «Boris Kidrič», *Vinča Belgrade* (Yougoslavie)

Acta Haemat 47 58–63 (1972)

Changes in Eosinophil Granulocyte Kinetics in Severe Hypoxia

G Hudson, K N Chin and D J Moffatt

Department of Haematology, University of Sheffield Sheffield

Abstract Kinetics of eosinophil granulocytes have been studied in guinea pigs simultaneously exposed to severe hypoxia at a simulated altitude of 20 000 ft (6 096 m) by determining the percentages of blood eosinophils showing DNA labelling at intervals after an 8 hour course of tritiated thymidine injections Labelled eosinophils were detected at an earlier interval in hypoxic animals (36 h against 72 h in the controls) and a higher percentage labelling was found up to and including 108 h Thereafter, percentage labelling was generally similar to that of the controls The results are taken to indicate a reduction in transit time across the postmitotic marrow eosinophil pool and to be consistent with an increased rate of release of these cells into the circulation

Key Words
Autoradiography
Eosinophil kinetics
Guinea pig eosinophils
Hypoxia

In earlier investigations [3, 8, 11] a striking increase was observed in the eosinophil granulocyte count of the peripheral blood of guinea pigs during the first week or more of exposure to severe hypoxia Quantitative studies showed that the peripheral eosinophilia was accompanied by a depletion of marrow eosinophil numbers and suggested that at least part of the explanation was that large numbers of mature or almost mature eosinophils were released from the bone marrow into the blood in the first few days of severe hypoxia. To a lesser extent, the other types of granulocyte seemed also to be affected

In an attempt to throw further light on the kinetics of eosinophil granulocytes in these circumstances, the emergence of DNA-labelled cells into the circulation has been studied following a course of tritiated thymidine injections which has been shown to lead to near 100% labelling of blood eosinophils in normal animals [7]

Material and Methods

The work was carried out on 10 healthy male albino guinea pigs of the Dunklin Hartley strain. Each weighed approximately 400 g at the beginning of the experiment and the resting blood eosinophil count was within the normal range [4]. For 1 week prior to the main part of the experiment, each animal was kept in a decompression chamber [10], with the decompression pump running continuously, but with normal atmospheric pressure being maintained. Each animal was removed twice daily for weighing while the chamber was cleaned and the food and drinking water replenished.

For the main part of the experiment, which began with the injections of tritiated thymidine (see below), 4 animals continued to be maintained in the decompression chamber at normal atmospheric pressure and under the same conditions as before. These animals will be referred to as the 'controls'. The other 6 were similarly maintained but at a barometric pressure of 3.5 Torr, corresponding to an altitude of approximately 20,000 ft (6,096 m). These animals will be referred to as 'hypoxic' or 'severely hypoxic'. In the case of 3 animals (sub-group A), decompression was commenced 16 h prior to the first injection and in the case of the other 3 (sub-group B) it was commenced immediately after the last injection of tritiated thymidine. Exposure to lowered barometric pressure was continuous, except for 2 periods of about 20 min each day when the chamber was returned to atmospheric pressure to enable the animal to be examined and the chamber serviced as described above.

Each animal received 5 intraperitoneal injections of tritiated thymidine in doses of 1 μCi per g of body weight. The injections were given at 2 hour intervals, the first being given at 09.00 and the last at 17.00 h. Smears of ear vein blood were made at intervals of 12 h over the period 36–180 h after the first injection. On the last day of the experiment, the direct blood eosinophil count was repeated.

The smears were air-dried and fixed immediately in methyl alcohol for 5 min. They were subsequently coated with Ilford K5 fine grain emulsion and exposed in light proof boxes for 4 days before developing, fixing and washing. They were then stained through the fixed emulsion with MacNeal's tetrachrome stain. A small number of the smears had to be discarded because of a technical mishap (see Results).

In the smears obtained from each animal at each interval, up to 50 eosinophils were identified, grains over the nucleus and over an adjacent background area being counted by a method described previously [7]. From the results obtained, it appeared that the presence of 3 or more grains over an eosinophil nucleus could be taken as indicating DNA labelling.

Results

The results are summarised in table I. Except for the figures in brackets, the mean values ± standard deviations relate to the percen-

Table I Percentage DNA labelling of blood eosinophils

Hours	Controls %	Hypoxia %	Hypoxia vs controls t	Hypoxia sub-groups A %	B %
36	0 (0)	5±5 (1±2)	2 0	5±6	4±4
48	0 (0)	9±5 (3±3)	3 3[1]	14±3	5±2
60	0 (0)	22±10 (6±6)	4 2[1]	21±8	23±14
72	12±7 (1±2)	31±18 (9±11)	2 0	27±20	36±17
84	7±9 (1±2)	55±6 (23±9)	6 0[1]	53±2	57±8
96	24±12 (7±9)	53±17 (20±23)	2.9[1]	56±4	49±27
108	32±16 (10±4)	70±15 (28±18)	3 9[1]	67±13	73±18
120	71±11 (32±8)	71±17 (14±11)	0	65±17	78±19
132	55±40 (21±27)	82±13 (42±31)	1 5	83±11	82±20
144	77±25 (16±21)	84±12 (34±24)	0 6	87±2	80±23
156	96±4) (13±8)	93±5 (35±25)	0 7	96±1	89±7
168	96±6 (20±18)	91±9 (26±33)	0 9	95±9	85±3
180	72±24 (17±23)	89±14 (22±29)	1 3	99±2	74±3

[1] Statistical significance

tages of blood eosinophils showing 3 or more grains over the nucleus The t values are those of the standard error test for small samples and relate to the differences between the means for the control and hypoxic animals at each interval after tritiated thymidine administration, statistical significance at the 0 05 level is indicated The values in brackets re-

late to the percentages of eosinophils with more than 40 grains over the nucleus The results of the 2 hypoxic sub-groups are also shown separately, it should be noted that for sub-group B, there were only 2 observations at each interval from 120 h onwards, as the preparations from the third animal were unsatisfactory (see above)

Other findings In the control animals, the direct counts of eosinophils per mm^3 of blood were almost the same at the end of the experiment as they had been at the beginning, the average final count being 0 95 times the initial count ($\pm$ a standard deviation of 0 1 times) In the hypoxic animals the average final count was 8 6 times the initial value ($\pm$6.3 times) Examination of the smears of the hypoxic animals indicated that a marked eosinophilia was present throughout.

Discussion

The findings in the control animals were the same as reported earlier [7], namely that labelled eosinophils were first detected at 72 h and that maximal percentage labelling was first observed at 156 h. These two findings may be taken as providing a rough estimate of the minimal and maximal transit time respectively through the postmitotic marrow pool of eosinophils [7] In the hypoxic animals (table I), labelled eosinophils were detected earlier, a small number being already present at 36 h. Significantly higher percentages of labelled eosinophils were observed at 48, 60, 84, 96, and 108 h, but thereafter the percentage labelling was generally similar to that of the controls, a figure approaching 100% not being observed until 156 h. The results in the 2 hypoxic sub-groups were in general agreement with one another and would support similar conclusions

When the percentages of heavily-labelled eosinophils are considered (table I), it may be noted that these also were detected earlier in the hypoxic animals than in the controls, but caution must be exercised in drawing other conclusions because of the smaller numbers counted and the relatively large error terms

The differences between the hypoxic and control groups may be interpreted as indicating a reduction in both the minimum and the average transit times of eosinophils across the postmitotic marrow pool in the early stages of severe hypoxia This would imply that, as suggested in an

earlier investigation [3] eosinophils are released into the circulation at an increased rate

In attempting to understand the mechanism by which such an increased rate of eosinophil release could be mediated, it must be borne in mind that no evidence has been found [3] that the loss of eosinophils (and other granulocytes) from the marrow in the early stages of severe hypoxia is followed by active proliferation of the marrow precursors, on the contrary, the evidence indicates a decrease in production [11] In this the eosinophil response differs markedly from that seen in immunological reactions For example, in the response to injection of Trichinella spiralis larvae, in which a soluble plasma factor (eosinophil releasing factor) is thought to be involved, the mobilization of eosinophils is followed by a striking proliferation of precursors in the bone marrow [12, 13] In view of this difference, it seems reasonable to suggest that in the circumstances of the present observations, local factors in the bone marrow itself may be important in bringing about an increased rate of granulocyte release In this connection, marrow blood flow could play a part The rate of blood flow has been shown to influence leucocyte release from the marrow [2] and the increase in marrow vascularity in severe hypoxia is such that it can be appreciated with the naked eye [4] Another factor which may be important is a concomitant change in the micro-environment of the marrow cells The latter possibility has also been raised by observations indicating that in mice subjected to hypoxia or other forms of erythropoietic stress, the size of the committed myeloid stem-cell compartment in the marrow is reduced while that in the spleen is increased [9] It seems reasonable to think that such local factors could influence the different granulocytic cell lines to a different degree

The earlier investigations [3, 11] suggested that in addition to the changes discussed above, there was a decrease in the turnover of eosinophils in the blood during severe hypoxia In the present observations, the increased time interval between the initial appearance of labelled cells in blood and maximal percentage labelling in the hypoxic animals could also be taken to support this interpretation Under normal circumstances, most of the circulating granulocytes are thought to leave the blood at random presumably in response to tissue demands, while selective removal of senescent cells plays only a minor role [1] If as postulated above local factors in the marrow were important in determining an increased rate of eosinophil release into the circulation a lengthening

of turnover time in the blood might be explicable in terms of the supply of eosinophils to the blood being in excess of tissue demands

Acknowledgements The authors are indebted to Prof E. K. BLACKBURN for helpful comments and to Mrs HELEN PILLING for preparation of the manuscript

References

1 CRONKITE, E. P Kinetics of granulocytopoiesis. Nat Cancer Inst. Monogr *30* 51–62 (1969)

2 DORNFEST, B S, LOBUE, J HANDLER, E. S, GORDON A. S., and QUASTLER, H Mechanisms of leukocyte production and release I Factors influencing leukocyte release from isolated perfused femora. Acta haemat., Basel *28* 42–60 (1962)

3 GRANT J B F and HUDSON G A quantitative study of blood and bone marrow eosinophils in severe hypoxia. Brit. J Haemat. *17* 121 127 (1969)

4 HUDSON G Effect of hypoxia on bone marrow volume Brit. J Haemat. *4* 239–248 (1958)

5 HUDSON G Eosinophil populations in blood and bone marrow of normal guinea pigs Amer J Physiol *198* 1171–1173 (1960)

6 HUDSON G Quantitative study of eosinophil granulocytes. Sem Hemat *5* 166–186 (1968)

7 HUDSON, G CHIN K. N., and MOFFATT, D J Marrow reserve of eosinophils estimation of transit time in normal guinea pigs. Cell Tissue Kin. (in press).

8 HUDSON G, SMITH N C. W, WILSON, R. S and YOFFEY J M Eosinophil granulocytes and hypoxia. Nature, Lond. *213* 818–819 (1967)

9 RICKARD K. A., RENCRICCA, N J SHADDUCK, R K., MONETTE, F C. HOWARD D E., GARRITY M., and STOHLMANN F Myeloid stem cell kinetics during erythropoietic stress Brit J Haemat. *21* 537 547 (1971)

10 ROGERS, A. F.. A high altitude chamber for small animals J Anat., Lond *102* 160–161 (1968)

11 SIBLEY Y D L. and HUDSON, G Eosinophil leucocytes and recovery from severe hypoxia Acta haemat., Basel *43* 31–39 (1970)

12 SPRY, C. J F Mechanism of eosinophilia. V Kinetics of normal and accelerated eosinopoiesis. Cell Tissue Kin *4* 351–364 (1971)

13 SPRY C. J F Mechanism of eosinophilia. VI Eosinophil mobilization. Cell Tissue Kin *4* 365–374 (1971)

Authors addresses Dr G HUDSON and Dr K.N CHIN Faculty of Medicine University of Sheffield, *S10 2TN Sheffield* (England), Dr D J MOFFATT, Department of Anatomy University of Iowa, *Iowa City* (USA)

Transplantation Reviews, vol 6 Munksgaard Copenhagen 1971 Dan Kr 60.–

This latest volume of the series 'Transplantation Reviews' deals with antigens pertinent to transplantation on the one side and the antigens characteristic for different kinds of lymphocytes on the other In the first paper of the volume, KLEIN and SHREFFLER discuss the inheritance of the histocompatibility antigens of mice and man In the last paper, REISFELD and KAHAN give an account of the long lasting work on the purification of soluble histocompatibility antigens Our understanding of the histocompatibility system is still rudimentary so that biochemist and geneticist cannot meet yet as in the field of immunoglobulin research But the new developments demonstrated in the two papers render it likely that this will happen within a few years

The two middle papers deal with functional lymphocyte populations as characterized by different antisera WAKSMAN accounts for the work of his group in rats using heterologous antisera RAFF discusses mice with emphasis on θ and TL antigens and immunoglobulin receptors

The volume contains papers on topics in two fields which are highly active at the present time It is useful for every immunologist T L. VISCHER *Basel*

CROSS MAKELA and KOSUNEN (eds) **Cell Interaction and Receptor Antibodies in Immune Responses.** Academic Press London 1971 £ 7 00 US $ 21 00

The Third Sigrid Jusélius Symposium was held in Helsinki in June 1970 and the resulting book appears one year later This is a book about lymphocytes, their classification, characteristics and interaction with other cells The contents are very well organized with chapters following each other in logical order There is a short but informative introduction by the editors who emphasize problems and highlights and who develop a thread which helps through the papers One great concern are the two classes of lymphocytes the thymus dependent or T lymphocytes and the bone marrow or bursa derived B lymphocytes Both are necessary for production of antibody which is produced by the latter How to recognize them is dealt with in one chapter, how they are triggered in another how they collaborate with each other or with other cells is dealt with in several other chapters. Althoug it took a year before the book has appaered the papers give information full of actuality and point out the direction where research will move No other book is available with so much information on the topic It can be highly recommended T L. VISCHER *Basel*

Acta Haemat 48 65-71 (1972)

Daily Requirements and Physiologic Efficiency of Intrinsic Factor in Man[1]

J J TWOMEY, NELDA D VILLANUEVA, ARLINE H LAUGHTER, S J DANNA, T P. HAYNIE and M F. JAHNS

The Veterans Administration Hospital Baylor College of Medicine and The University of Texas M D Anderson Hospital and Tumor Institute at Houston Houston, Tex

Abstract Absorption from daily dietary equivalents of vitamin B_{12} by patients with pernicious anemia was significant but was not nutritionally adequate Increasing the amount of either vitamin B_{12} or exogenous intrinsic factor (IF) ingested by these patients increased vitamin B_{12} absorption To absorb 1 μg from a 20-μg dose of vitamin B_{12} the patients needed enough IF to bind 5 to 10 μg of vitamin B_{12}. Hog IF was found to mediate absorption from physiological doses of vitamin B_{12} as efficiently as human IF

Key Words
Hog intrinsic factor
Intrinsic factor requirements
Pernicious anemia
Total body counter
Vitamin B_{12} absorption

It is desirable that man absorbs at least 1 μg of vitamin B_{12} each day Intrinsic factor (IF) is needed to absorb enough vitamin B_{12} from normal dietary sources to maintain nutritional balance The literature mostly concerns absorption from tracer as opposed to physiological doses of vitamin B_{12} This report concerns studies in man of the amount of IF needed to absorb nutritionally adequate amounts of vitamin B_{12} from doses comparable to the vitamin B_{12} content in a normal diet The efficiency with which human and hog IF mediated vitamin B_{12} absorption was also compared

[1] Supported by V A Research Funds

Materials and Methods

Studies were on patients with pernicious anemia (PA) in remission IF secretory failure was demonstrated by II stage SCHILLING tests [18] and by failure to detect IF in betazole-stimulated depepsinized aspirates of their gastric juice [21] A radioimmunoassay was used to measure IF and to detect IF vitamin B_{12} blocking antibody [7] Human IF was from a pool of depepsinized and lyophilized betazole stimulated gastric juice Hog IF was a mucosal extract (Lederle WLS 942)

Dietary vitamin B_{12} was restricted to 0.5 μg per day on each test day and the day preceding it Test doses were ingested after an overnight fast and contained 0 5 μCi ^{60}Co vitamin B_{12} Whole body counting was performed before and 1 h after dosing and again 7 days later Urinary excretion of ingested ^{60}Co-vitamin B_{12} was also measured The whole-body counter included a chamber with 8 in steel walls an 8 in × 4 in NaI crystal and a 512 channel analyzer Patients were counted for 200 sec in each of 4 prone and 4 supine patient detector geometries Percent absorption varied less than 1% and was similar when measured 7 and 14 days after dosing Stools contained negligible radioactivity when body retention was counted

Results

The capacity of human IF to bind vitamin B_{12} *in vitro* was compared with its capacity to mediate vitamin B_{12} absorption *in vivo* by 2 patients We measured absorption from 20-μg doses of vitamin B_{12} which approximates the vitamin B_{12} content in an average day s diet [5] Neither patient absorbed more than 0 2 μg of vitamin B_{12} without exogenous IF (fig 1) There was a logarithmic relationship between the vitamin B_{12} binding capacity of the IF *in vitro* and the amount of vitamin B_{12} absorption mediated by this IF The data indicate that the patients required that amount of IF which could bind 4 4 and 8 2 μg of vitamin B_{12} in order to absorb 1 μg from 20-μg doses of vitamin B_{12} The patient with less efficient IF-mediated vitamin B_{12} absorption was shown to have IF-vitamin B_{12} blocking antibody in his gastric juice

The effect of varying the size of vitamin B_{12} doses upon IF-dependent and independent vitamin B_{12} absorption was evaluated A patient ingested vitamin B_{12} in doses ranging from 2 5 to 80 μg without, and later with that amount of IF capable of binding 15 μg of vitamin B_{12} When the dose of vitamin B_{12} ingested without exogenous IF was increased from 20 to 80 μg IF-independent vitamin B_{12} absorption increased from 0 2 to 0 6 μg (fig 2) IF-dependent vitamin B_{12} absorption was also enhanced when the amount of IF was ingested with increasing doses

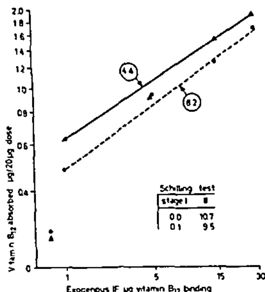


Fig 1 The relationship between available IF and absorption from 20 µg of vitamin B_{12} ▲ Patient without IF vitamin B_{12} blocking antibody ● Patient with IF vitamin B_{12} blocking antibody Values of least absorption were without exogenous IF

Table I A comparison between human and hog IF mediated vitamin B_{12} absorption

	% vitamin B_{12} absorption		
	1	2	3
Without exogenous IF	0.8	0.9	<1.0
With human IF (human blocking antibody)	22.1	8.0	5.5
With hog IF (human blocking antibody)	16.5	7.4	5.3
With hog IF (hog mucosal antibody)	14.0	4.0	4.2

of vitamin B_{12} There was a direct relationship between the amount of vitamin B_{12} ingested and the amount of IF mediated vitamin B_{12} absorption

The effectiveness with which hog IF mediated vitamin B_{12} absorption was compared with the effectiveness of human IF We measured absorp-

Materials and Methods

Studies were on patients with pernicious anemia (PA) in remission IF secretory failure was demonstrated by II stage SCHILLING tests [18] and by failure to detect IF in betazole stimulated depepsinized aspirates of their gastric juice [21] A radio immunoassay was used to measure IF and to detect IF vitamin B_{12} blocking antibody [7] Human IF was from a pool of depepsinized and lyophilized betazole stimulated gastric juice Hog IF was a mucosal extract (Lederle WES 942)

Dietary vitamin B_{12} was restricted to 0.5 μg per day on each test day and the day preceding it Test doses were ingested after an overnight fast and contained 0.5 μCi ^{60}Co vitamin B_{12} Whole body counting was performed before and 1 h after dosing and again 7 days later Urinary excretion of ingested ^{60}Co-vitamin B_{12} was also measured The whole body counter included a chamber with 8 in steel walls an 8 in × 4 in NaI crystal and a 512 channel analyzer Patients were counted for 200 sec in each of 4 prone and 4 supine patient-detector geometries Percent absorption varied less than 1% and was similar when measured 7 and 14 days after dosing Stools contained negligible radioactivity when body retention was counted

Results

The capacity of human IF to bind vitamin B_{12} *in vitro* was compared with its capacity to mediate vitamin B_{12} absorption *in vivo* by 2 patients We measured absorption from 20 μg doses of vitamin B_{12} which approximates the vitamin B_{12} content in an average day s diet [5] Neither patient absorbed more than 0 2 μg of vitamin B_{12} without exogenous IF (fig 1) There was a logarithmic relationship between the vitamin B_{12} binding capacity of the IF *in vitro* and the amount of vitamin B_{12} absorption mediated by this IF The data indicate that the patients required that amount of IF which could bind 4 4 and 8 2 μg of vitamin B_{12} in order to absorb 1 μg from 20 μg doses of vitamin B_{12} The patient with less efficient IF-mediated vitamin B_{12} absorption was shown to have IF vitamin B_{12} blocking antibody in his gastric juice

The effect of varying the size of vitamin B_{12} doses upon IF-dependent and independent vitamin B_{12} absorption was evaluated A patient ingested vitamin B_{12} in doses ranging from 2 5 to 80 μg without, and later with that amount of IF capable of binding 15 μg of vitamin B_{12} When the dose of vitamin B_{12} ingested without exogenous IF was increased from 20 to 80 μg IF independent vitamin B_{12} absorption increased from 0 2 to 0 6 μg (fig 2) IF-dependent vitamin B_{12} absorption was also enhanced when the amount of IF was ingested with increasing doses

tional to vitamin B_{12} intake [4] Vitamin B_{12} deficiency should there fore, develop quicker in patients who ingest inadequate amounts of vitam in B_{12} than in patients with PA However, reabsorption of biliary vitamin B_{12} helps patients capable of IF secretion conserve vitamin B_{12}

One μg of vitamin B_{12} given intramuscularly each day usually corrects megaloblastic anemia due to vitamin B_{12} deficiency [10] This amount cannot be absorbed from a normal diet without IF It has proven possible to correct nutritional vitamin B_{12} deficiency with a diet that contains 1 5 μg of vitamin B_{12} per day [20] about 60% of which is ab sorbed if IF secretion and ileal mucosa are normal [12, 13] Our data suggest that, in the presence of IF, absolute absorption is greater from large doses of vitamin B_{12} absorption is more efficient from small doses Greater IF mediated vitamin B_{12} absorption could be due to (1) a better concentration of vitamin B_{12} to bind with IF within the intestine, or (2) reutilization of IF that had bound earlier with other molecules of vitamin B_{12} to ileal receptor sites [11]

Our data suggest that the amount of human IF required to bind 5 to 10 μg of vitamin B_{12} is needed to mediate the absorption of 1 μg from 20 μg doses of vitamin B_{12} This may approximate the amount of IF needed each day to maintain nutritional equilibrium This is at least 10 times greater than the IF requirement to correct the SCHILLING test on pa tients with PA [1] The difference probably reflects differences in both dose size and the amounts of vitamin B_{12} absorbed Healthy adults can release enough IF to bind 2 to 28 μg of vitamin B_{12} within 2 h following pharmacologic stimulation [2 16, 21] Our data suggest that IF mediated vitamin B_{12} absorption approaches optimal efficiency in the presence of physiologic IF secretion

These results may represent a slight underestimate of daily IF require ments because (1) The vitamin B_{12} and IF were ingested together, this provided optimal opportunity for IF vitamin B_{12} interaction (2) IF is probably degraded at a reduced rate in patients with atrophic gastritis (3) IF autoantibody interferes with vitamin B_{12} absorption [17] The larger IF requirements by one patient (fig 1) may have been due to the IF au toantibody in his own gastric secretions

There are antigenic differences between human and hog IF [6, 14] In contrast to RAMSEY and HERBERT [14] who used a different mucosal ex tract we found that human IF vitamin B_{12} blocking autoantibody inhib ited 27% of vitamin B_{12} binding by WES 942 hog mucosal extract Hog IF as assayed using human autoantibody was approximately as efficient

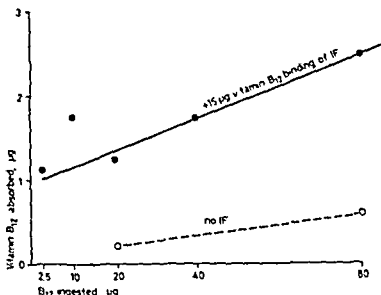


Fig 2 The relationship between the amount of vitamin B_{12} ingested and vitamin B_{12} absorbed

tion from 10-µg doses of vitamin B_{12} that were ingested with doses of human and then hog IF that could bind 5 µg of vitamin B_{12}. We first attempted to determine IF-specific vitamin B_{12} binding. Human IF-vitamin B_{12} blocking autoantibody inhibited 88% of vitamin B_{12} binding by the human gastric juice and 27% of binding by the hog mucosal extract. Rabbit antiserum to hog mucosal extract blocked all binding by the hog mucosal extract. Doses of hog and human IF, as determined using human autoantibody, mediated comparable absorption in 2 patients (table I), hog IF was slightly less effective in a third patient. In all 3 instances, a dose of hog IF, as determined using rabbit antiserum to hog mucosal extract (which suggested that binding by this extract was not 'IF-specific') mediated less absorption than either human or hog IF when 'IF-specific' binding was determined using human autoantibody.

Discussion

The normal turnover rate of vitamin B_{12} by man is about 2.5 µg per day [8, 9]. *Our data indicate that significant, but nutritionally inadequate, amounts of dietary* vitamin B_{12} are absorbed without IF, this is propor-

tional to vitamin B_{12} intake [4] Vitamin B_{12} deficiency should, therefore, develop quicker in patients who ingest inadequate amounts of vitamin B_{12} than in patients with PA However, reabsorption of biliary vitamin B_{12} helps patients capable of IF secretion conserve vitamin B_{12}

One μg of vitamin B_{12} given intramuscularly each day usually corrects megaloblastic anemia due to vitamin B_{12} deficiency [10] This amount cannot be absorbed from a normal diet without IF It has proven possible to correct nutritional vitamin B_{12} deficiency with a diet that contains 1 5 μg of vitamin B_{12} per day [20], about 60% of which is absorbed if IF secretion and ileal mucosa are normal [12, 13] Our data suggest that, in the presence of IF, absolute absorption is greater from large doses of vitamin B_{12}, absorption is more efficient from small doses Greater IF-mediated vitamin B_{12} absorption could be due to (1) a better concentration of vitamin B_{12} to bind with IF within the intestine, or (2) *reutilization of IF that had bound earlier with other molecules of vitamin* B_{12} to ileal receptor sites [11]

Our data suggest that the amount of human IF required to bind 5 to 10 μg of vitamin B_{12} is needed to mediate the absorption of 1 μg from 20 μg doses of vitamin B_{12} This may approximate the amount of IF needed each day to maintain nutritional equilibrium This is at least 10 times greater than the IF requirement to correct the SCHILLING test on patients with PA [1] The difference probably reflects differences in both dose size and the amounts of vitamin B_{12} absorbed Healthy adults can release enough IF to bind 2 to 28 μg of vitamin B_{12} within 2 h following pharmacologic stimulation [2, 16 21] Our data suggest that IF-mediated vitamin B_{12} absorption approaches optimal efficiency in the presence of physiologic IF secretion

These results may represent a slight underestimate of daily IF requirements because (1) The vitamin B_{12} and IF were ingested together, this provided optimal opportunity for IF vitamin B_{12} interaction (2) IF is probably degraded at a reduced rate in patients with atrophic gastritis (3) IF autoantibody interferes with vitamin B_{12} absorption [17] The larger IF requirements by one patient (fig 1) may have been due to the IF autoantibody in his own gastric secretions

There are antigenic differences between human and hog IF [6, 14] In contrast to RAMSEY and HERBERT [14] who used a different mucosal extract, we found that human IF vitamin B_{12} blocking autoantibody inhibited 27% of vitamin B_{12} binding by WES 942 hog mucosal extract. Hog IF, as assayed using human autoantibody, was approximately as efficient

as human IF at mediating vitamin B_{12} absorption by our patients SCHWARTZ [19] made similar observations using tracer doses of vitamin B_{12} However, REIZENSTEIN et al [15] found that hog mucosa lowered absorption from tracer doses of vitamin B_{12} by healthy subjects Much of the vitamin B_{12} bound by hog mucosal extract does not appear to be IF specific and may interfere with endogenous IF Our results suggest that antisera made to mucosal extracts blocks all or most vitamin B_{12} binding and does not specifically identify IF binding

Acknowledgements We are indebted to LEON ELLENBOGEN M D for WES 942 hog mucosal extract to VICTOR HERBERT M D for rabbit antiserum to hog mucosal extract to LEONARD GOLDBERG M D., for assaying for autoantibody in gastric juice and to Ms DONNA DICKINSON for secretarial assistance

References

1 ARDEMAN S and CHANARIN I Assay of gastric IF in the diagnosis of Addisonian PA Brit J Haemat *11* 305 (1965)
2 ARDEMAN S CHANARIN I and DOYLE J C Studies on secretion of gastric intrinsic factor in man Brit med J *ii* 600 (1964)
3 ASHWORTH L A E. STRICKLAND M B KOO N C., and TAYLOR K B Effect of pH on intrinsic factor and nonintrinsic factor vitamin B_{12} binding in human gastric juice neutralized *in vivo* Gastroenterology *57* 506 (1969)
4 BERLIN H BERLIN R., and BRANTE, G Oral treatment of pernicious anemia with high doses of vitamin B_{12} without intrinsic factor Acta med scand *184* 247 (1968)
5 CHUNG A S PEARSON W N DARBY W J MILLER O N and GOLDSMITH G A Folic acid vitamin B_6 pantothenic acid and vitamin B_{12} in human dietaries. Amer J clin Nutr *9* 573 (1961)
6 COOPER B A and LOWENSTEIN L Acquired resistance to hog intrinsic factor Lancet *i* 1057 (1963)
7 GOTTLIEB C LAU K S WASSERMAN L R and HERBERT V Rapid charcoal assay for intrinsic factor (IF) gastric juice unsaturated B_{12} binding capacity antibody to IF and serum unsaturated B_{12} binding capacity Blood *25* 875 (1965)
8 HEINRICH H C Metabolism of Co^{60} and Co^{58} labeled vitamin B_{12} in humans and rats in Radioactivity in man chapt 22, p 290 (Thomas Springfield 1965)
9 HERBERT V Nutritional requirements for vitamin B_{12} and folic acid Amer J clin Nutr *21* 743 (1968)
10 HEYSSEL R M BOZIAN R C DARBY W J, and BELL, M C Vitamin B turnover in man The assimilation of vitamin B_{12} from natural foodstuff by man and estimates of minimal daily dietary requirements Amer J clin Nutr *18* 176 (1966)

11 HINES J D, ROSENBERG, A, and HARRIS, J W IF mediated radio B_{12} uptake in sequential incubation studies using everted sacs of guinea pig small intestine evidence that IF is not absorbed into the intestinal cell Proc Soc exp Biol, N Y *129* 653 (1968)
12 NAVERSTEN, Y, LIDEN K, STAHLBERG, K G, and NORDEN, A The study of 57 Co-vitamin B_{12} absorption using a whole bodycounter Phys Med Biol *14* 441 (1969)
13 POLLYCOVE, M and APT, L Absorption, elimination, and excretion of orally administered vitamin B_{12} in normal subjects and in patients with pernicious anemia New Engl J Med *255* 207 (1956)
14 RAMSEY, C and HERBERT, V Dialysis assay for intrinsic factor and its antibody demonstration of species specificity of antibodies to human and hog intrinsic factor J Lab clin Med *65* 143 (1965)
15 REIZENSTEIN P G, CRONKITE, E. P., and COHN, S H Measurement of absorption of vitamin B_{12} by whole body gamma spectrometry Blood *18* 95 (1961)
16 RODBRO P, CHRISTIANSEN, P M., and SCHWARTZ, M Intrinsic factor secretion in stomach diseases Lancet *ii* 1200 (1965)
17 ROSE, M S and CHANARIN, I Intrinsic factor antibody and absorption of vitamin B_{12} in pernicious anemia Brit med J *i* 25 (1971)
18 SCHILLING, R F Intrinsic factor studies II The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12} J Lab clin Med *42* 860 (1953)
19 SCHWARTZ, M Antibody to intrinsic factor Scand J clin Lab Invest *19* suppl., vol 95, p 19 (1967)
20 STEWART, J S, ROBERTS, P L D, and HOFFBRAND, A V Response of dietary vitamin B_{12} deficiency to physiological oral doses of cyanocobalamin Lancet *ii* 542 (1970)
21 TWOMEY, J J, LAUGHTER, A H, and JORDAN, P H., jr Studies into human IF secretion Amer J digest Dis *16* 1075 (1971)

Author's address Dr JEREMIAH J TWOMEY, 2002 Holcombe Boulevard, *Houston TX 77031* (USA)

as human IF at mediating vitamin B_{12} absorption by our patients SCHWARTZ [19] made similar observations using tracer doses of vitamin B_{12} However, REIZENSTEIN et al [15] found that hog mucosa lowered absorption from tracer doses of vitamin B_{12} by healthy subjects Much of the vitamin B_{12} bound by hog mucosal extract does not appear to be IF-specific and may interfere with endogenous IF Our results suggest that antisera made to mucosal extracts blocks all or most vitamin B_{12} binding and does not specifically identify IF binding

Acknowledgements We are indebted to LEON ELLENBOGEN M D, for WLS 942 hog mucosal extract, to VICTOR HERBERT, M D, for rabbit antiserum to hog mucosal extract, to LEONARD GOLDBERG M D, for assaying for autoantibody in gastric juice, and to Ms DONNA DICKINSON for secretarial assistance

References

1 ARDEMAN S and CHANARIN I Assay of gastric IF in the diagnosis of Addisonian PA Brit J Haemat *11* 305 (1965)
2 ARDEMAN S, CHANARIN I, and DOYLE, J C Studies on secretion of gastric intrinsic factor in man Brit med J *ii* 600 (1964)
3 ASHWORTH L A E STRICKLAND, M B, KOO N C, and TAYLOR K B Effect of pH on intrinsic factor and nonintrinsic factor vitamin B_{12} binding in human gastric juice neutralized *in vivo* Gastroenterology *57* 506 (1969)
4 BERLIN H BERLIN R, and BRANTE, G Oral treatment of pernicious anemia with high doses of vitamin B_{12} without intrinsic factor Acta med scand *184* 247 (1968)
5 CHUNG A S PEARSON W N DARBY, W J, MILLER O N, and GOLDSMITH G A Folic acid vitamin B_6 pantothenic acid and vitamin B_{12} in human dietaries Amer J clin Nutr *9* 573 (1961)
6 COOPER B A and LOWENSTEIN L Acquired resistance to hog intrinsic factor Lancet *i* 1057 (1963)
7 GOTTLIEB C, LAU K S WASSERMAN I R, and HERBERT, V Rapid charcoal assay for intrinsic factor (IF) gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity Blood *25* 875 (1965)
8 HEINRICH H C Metabolism of Co^{60} and Co^{58} labeled vitamin B_{12} in humans and rats in Radioactivity in man chapt 22 p 290 (Thomas Springfield 1965)
9 HERBERT V Nutritional requirements for vitamin B_{12} and folic acid Amer J clin Nutr *21* 743 (1968)
10 HEYSSEL R M BOZIAN R C DARBY W J, and BELL, M C Vitamin B turnover in man The assimilation of vitamin B_{12} from natural foodstuff by man and estimates of minimal daily dietary requirements Amer J clin Nutr *18* 176 (1966)

Table 1 Main clinical findings in the 4 presented patients

Patient	Age	Sex	Erythrocytes, mill mm³	Hematocrit %	Hemoglobin, g%	Thrombocytes, mm³	Leucocytes mm³	PMN and bandformed neutrophils mm³	Neutrophil precursors mm³	Eosinophils mm³	Lymphocytes mm³	Monocytoid cells mm³	Erythroblasts, mm³	Plasma cells mm³	Hepatomegalia	Splenomegalia	Alkaline leukocyte phosphatase	Duration from begin of symptoms to end of observation
1	42	F	1.8		5.2	12 000	23 850	235			2 115	21 500			–	–	45	6 weeks
2	67	M		33	10.5	32,000	22,200	11,320		290	2,750	7 840			–	+	65	4 months
3	66	F		24	9.0	34 000	7 200	252	900		756	5 004	216	72		+	60	8 weeks
4	66	M	1.9		7.2	27 000	10 500	1 785		105	945	7 665			+ +	+	5	4 weeks

– = Absent or normal + = slightly enlarged, + + = markedly enlarged

Acta Haemat 48 72-88 (1972)

Atypical (Monomyelocytic) Myelogenous Leukemia

Cytochemical, Electron Microscopic, and Biochemical Investigations

F Schmalzl, D Huhn, H Asamer, K Abbrederis
and H Braunsteiner

Medizinische Klinik der Universitat Innsbruck
(Vorstand Prof Dr H Braunsteiner)
and Institut für Hamatologie der Gesellschaft fur Strahlen und Umweltforschung
Assoziation mit Euratom und Lehrstuhl für Innere Medizin
speziell Hamatologie der Universitat München (Vorstand Prof Dr W Stich)

Abstract Four cases of acute myelogenous leukemia characterized by peculiar leukemic cell populations are presented The cytochemical, ultrastructural, biochemical and functional features observed in these leukemic cells suggested a similar pathological differentiation trend which apparently led to the combination in a single cell population of features characteristic of both monocytes and myelocytes or promyelocytes

Key Words
Atypical myelogenous leukemia
Cytochemistry
Electron microscopy
Monomyelocytic leukemia

The application of special investigations to the study of leukemic or neoplastic blood and hematopoietic cells yielded new informations about their pathological differentiation Out of about 250 cases of first diagnosed acute leukemia we observed 4 cases of atypical myelogenous leukemia showing a cytochemical pattern suggestive of a pathological differentiation tendency resulting in a cell type intermediate between monocytes on the one hand and both promyelocytes and myelocytes on the other [52] The cytochemical biochemical, electron microscopic, and some functional features of these particular cell populations will be presented

Materials and Methods

The most important clinical findings in the 4 patients are summarized in table I The bone marrow aspirations showed a preponderant immature leukemic cell

Table 1 Main clinical findings in the 4 presented patients

Patient	Age	Sex	Erythrocytes, mill mm³	Hematocrit, %	Hemoglobin, g%	Thrombocytes mm³	Leucocytes mm³	PMN and bandformed neutrophils mm³	Neutrophil precursors, mm³	Eosinophils mm³	Lymphocytes mm³	Monocytoid cells mm³	Erythroblasts mm³	Plasma cells, mm³	Hepatomegalia	Splenomegalia	Alkaline leukocyte phosphatase	Duration from begin of symptoms to end of observation
1	42	F	1.8		5.2	12,000	23 850	235			2,115	21 500			–	–	45	6 weeks
2	67	M		33	10.5	32 000	22 200	11 320		290	2,750	7 840			–	+	65	4 months
3	66	F		24	9.0	34 000	7 200	252	900		756	5 004	216	72		+	60	8 weeks
4	66	M	1.9		7.2	27 000	10 500	1,785		105	945	7 665			+ +	+	5	4 weeks

– = Absent or normal + = slightly enlarged, + + = markedly enlarged

population corresponding to the cells present in the peripheral blood The leukemic cells present in the bone marrow appeared slightly less mature than those in the peripheral blood

Cytochemical methods Blood and bone marrow smears from each patient were studied at least twice The following staining procedures were performed after appropriate pretreatment May Grünwald Giemsa PAS reaction [21] Sudan black B stain [55] peroxidase following either the method of KAPLOW [28] or of SCHAEFFER and FISCHER [45] naphthol AS D chloroacetate esterase (N AS Cl-esterase) [38] nonspecific esterase with naphthol AS I C acetate as substrate (N AS esterase) [32 48] to test the inhibition of N AS esterase sodium fluoride (NaF) was added to the incubation medium at the concentration of 1.5 mg/ml A double incubation technique permitted the demonstration of both NaF sensitive and resistant esterase activities on the same slide [48] Tests for N AS esterase and N AS Cl esterase were also combined on the same slide [31 48] Cytochemical acid phosphatase [10] naphthylamidase [2] and alkaline phosphatase [27] reactions were also performed Nuclei were stained with either the Feulgen reaction or with Mayers acid hemalum

Electron microscopical methods Heparinized blood samples from the cubital vein were centrifuged for 30 min at 1 900 g The supernatant plasma was then carefully removed and the sedimented blood cells were covered with 6% glutaraldehyde in 0 2 M phosphate buffer at a pH of 7.3-7 7 After another 30 min the partly fixed leukocyte layer covering the erythrocytes was removed and fixed again for 4 h in fresh sample of the same fixative After postfixation in osmic acid the leukocyte concentrate was embedded in Maraglas [58] The sections were stained with uranyl acetate and lead hydroxide [44]

Skin window experiments were performed on 3 of the 4 patients according to the previously published method [54] The cellular exudates were stained with May Grünwald Giemsa and with the cytochemical reactions for acid phosphatase and nonspecific esterase (N AS-esterase)

Quantitative lysozyme determination was repeatedly carried out on serum and urinary samples of 3 of the 4 patients using the lyso plate technique of OSSERMAN and LAWLOR [41] Purified human lysozyme (kindly supplied by E F OSSERMAN New York)[1] was used as a standard

Immunocytological demonstration of lysozyme in the leukemic cells was performed by the technique reported in a previous publication [5] Appropriate tests for assessing the specificity of the positive labeling were performed [7]

Results

Morphology Observations of the May Grünwald Giemsa stained blood and bone marrow smears (fig 1) of the 4 patients showed definite morphological differences in their cells The cells of patient 1 appeared to be rather large with a round or oval seldom indented nucleus containing one or two nucleoli The large cytoplasm was customarily round or slightly elongated showing a grey color and

[1] At this time we wish to thank to Dr E F OSSERMAN for his kindness

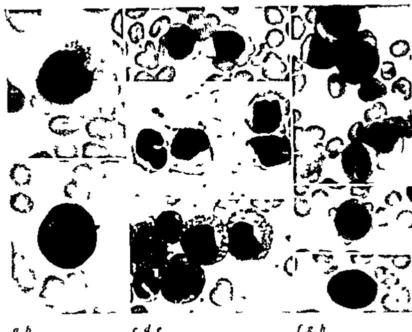


Fig 1 Comparison of rather mature appearing cells with cells having an immature appearance in patients with monomyelocytic leukemia May Grunwald Giemsa stain *a b* Peripheral blood of patient 1 × 1 400 *c d* Peripheral blood of patient 2 × 1 200 *e* Bone marrow of patient 2 × 1 200 *f g h* Peripheral blood of patient 3 showing less mature cells × 1,200

containing abundant coarse pink to reddish granules In patient 2 the leukemic cells were morphologically practically identical to normal monocytes although the cytochemical tests revealed high enzyme activities peculiar to the azurophilic granulation of the neutrophil granulocytes In patient 3 the leukemic cells were smaller than those of the other patients ranging between 12 and 15 μm in diameter and appearing to be rather immature The round nucleus contained few nucleoli The cytoplasm was medium sized and was preponderantly blue in color appearing to be greyish to slightly pink in a smaller perinuclear and sometimes paranuclear area Some pink or slightly reddish granules were also present In patient 4 the nuclei were oval kidney shaped or indented with scattered nucleoli the lightly greyish cytoplasm appeared rather homogenous containing only a small number of granules

Cytochemical investigations produced satisfactory results in both blood and bone marrow smears and preserved the nucleated cells ade

population corresponding to the cells present in the peripheral blood The leukemic cells present in the bone marrow appeared slightly less mature than those in the peripheral blood

Cytochemical methods Blood and bone marrow smears from each patient were studied at least twice The following staining procedures were performed after appropriate pretreatment May Grünwald Giemsa PAS reaction [21] Sudan black B stain [55] peroxidase following either the method of KAPLOW [28] or of SCHAEFER and FISCHER [45] naphthol AS D chloroacetate esterase (N AS-Cl esterase) [38] nonspecific esterase with naphthol AS I C acetate as substrate (N AS esterase) [32 48] to test the inhibition of N AS esterase sodium fluoride (NaF) was added to the incubation medium at the concentration of 1.5 mg/ml A double incubation technique permitted the demonstration of both NaF sensitive and resistant esterase activities on the same slide [48] Tests for N AS-esterase and N AS Cl esterase were also combined on the same slide [31 48] Cytochemical acid phosphatase [10] naphthylamidase [2] and alkaline phosphatase [27] reactions were also performed Nuclei were stained with either the Feulgen reaction or with Mayers acid hemalum

Electron microscopical methods Heparinized blood samples from the cubital vein were centrifuged for 30 min at 1 900 *g* The supernatant plasma was then carefully removed and the sedimented blood cells were covered with 6% glutaraldehyde in 0 2 M phosphate buffer at a pH of 7 3–7 7 After another 30 min the partly fixed leukocyte layer covering the erythrocytes was removed and fixed again for 4 h in fresh sample of the same fixative After postfixation in osmic acid the leukocyte concentrate was embedded in Maraglas [58] The sections were stained with uranyl acetate and lead hydroxide [44]

Skin window experiments were performed on 3 of the 4 patients according to the previously published method [54] The cellular exudates were stained with May Grünwald Giemsa and with the cytochemical reactions for acid phosphatase and nonspecific esterase (N AS-esterase)

Quantitative lysozyme determination was repeatedly carried out on serum and urinary samples of 3 of the 4 patients using the lyso plate technique of OSSERMAN and LAWLOR [41] Purified human lysozyme (kindly supplied by F F OSSERMAN New York)[1] was used as a standard

Immunocytological demonstration of lysozyme in the leukemic cells was performed by the technique reported in a previous publication [5] Appropriate tests for assessing the specificity of the positive labeling were performed [7]

Results

Morphology Observations of the May Grünwald Giemsa stained blood and bone marrow smears (fig 1) of the 4 patients showed definite morphological differences in their cells The cells of patient 1 appeared to be rather large with a round or oval seldom indented nucleus containing one or two nucleoli The large cytoplasm was customarily round or slightly elongated showing a grey color and

[1] At this time we wish to thank to Dr F F OSSERMAN for his kindness

quately The cytochemically demonstrated chemical constituents and the enzyme activities occurring within the leukemic cells of the four patients in question are summarized in figure 2 and are compared to some other types of myelogenous leukemias whose cytochemical patterns are quite similar to those of the respective normal blood and bone marrow cells

Monocytic leukemia is cytochemically characterized by a usually very prominent NaF-sensitive nonspecific N AS-esterase activity as well as by *the following common traits the faintly positive reactions of peroxidase* and N-AS Cl-esterase and the faintly positive Sudan black B staining (fig 3) [31, 34, 48, 50] Leukemic myeloblasts or paramyeloblasts stain only weakly with Sudan black B and exhibit a faintly traceable peroxidase activity [1, 19, 53] Chloracylesterase can only exceptionally be detected in these cells [1, 53] The latter enzyme reaction stains markedly or strongly the cytoplasm of promyelocytes and myelocytes which also contain very conspicuous peroxidase activity and abundant sudanophilia with Sudan black B [1, 19, 35, 53] Leukemic as well as normal promyelocytes show varying degrees of N-AS-esterase activity which, unlike that in monocytes, may be slightly or not at all inhibited by NaF [1, 20, 48, 53] (fig 2) The 4 presented cases form a further cytological type of myelogenous leukemia manifesting a differentiation tendency intermediate between that of monocytes and that of both promyelocytes and myelocytes The cytochemical features accounting for the peculiarity of these cells include varying but usually marked N-A-S-esterase activity, which may be similar to that of true monocytic leukemia The NaF-inhibition of this enzyme is also outstanding but is in general not as complete as that in monocytes Marked positive Sudan black B staining as well as conspicuous peroxidase activity are further traits of this particular type of leukemia [52] N-AS-Cl-esterase may be present to a lesser extent Among the different cases the staining intensity is seen to vary, but is still within the pattern just outlined

Cytochemical acid phosphatase activity is customarily related to the *presence of lysosomal granular structures and of the Golgi apparatus* [40] and therefore, along with other cytochemical staining results may *indicate* to some extent the maturation stage of some of the blood and bone marrow cells [3 8] From examination of the cytochemical patterns (fig 2) as well as from the morphological features it could be concluded that the leukemic cells of patients 1 and 2 were rather mature, whereas the leukemic cells in patients 3 and 4 reached a minor degree of maturation (fig 4)

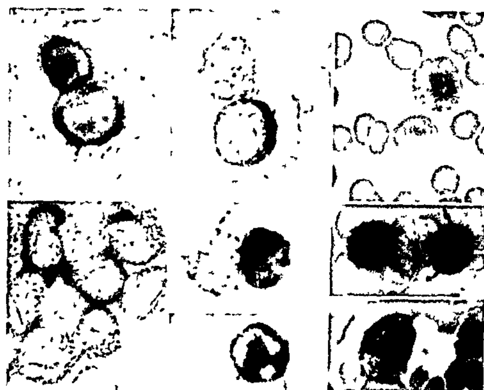


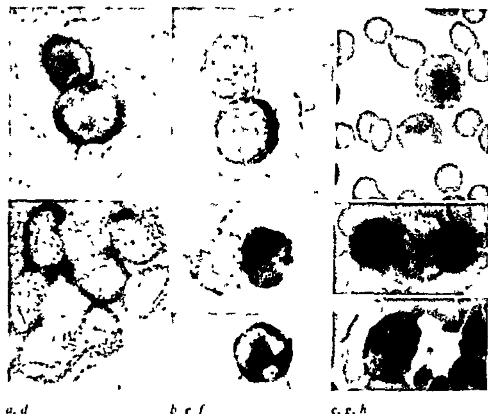
Fig 3 Comparison of the N AS esterase (a, d), the Sudan black B positivity (b, e f), and the peroxidase activity (c, g, h) of patient 1 (a b c) and patient 2 (d, e, f, g h) In figure 3b, c and g a weakly positive or negative leukemic mono cytoid cell is shown near a moderately or strongly stained cell In figure 3e, a strongly an weakly positive cell are compared to a strongly positive polymor phonuclear hil (f) × 1



Fig 4 Acid phosphatase Strong activity in the leukemic cells of patient 1 (a) and moderate and faint activity in the cells of patient 3 (b c arrows) Nuclear stain Mayer's hemalum × 1 000

big lobulated nuclei with large nucleoli The nuclear chromatin was evenly distributed showing a condensed rim along the nuclear envelope Nuclear pockets were rarely observed Some finger like cytoplasmic pro trusions were distributed around the cell surface An abundance of free ribosomes was present in the cytoplasm as well as some rather long er gastoplasmic strands The Golgi apparatus appeared well developed and sometimes multipolar shaped Numerous, often very large mitochondria distributed throughout the cytoplasm contained dense matrices and large intramitochondrial granules Rare intracytoplasmic bundles of fi brils were observed each fibril of which averaged about 100 A in diam eter The plentiful small electron dense granules (0 1 μm in diameter) were either uniformly distributed throughout the cytoplasm or arranged in groups Other, less electron dense granules of major size resembled the typical primary' and rarely the secondary granules of the neutro phil series Only in the leukemic cells of patient 2 a few Auer bodies were found No signs of phagocytosis occurred

Lysozyme For comparison the lysozyme levels of the urine and the serum of the 3 patients tested are shown in table II along with the lev els found in other types of leukemia In patients with acute myeloblastic leukemia the urinary lysozyme levels are usually low, whereas they may be high in some cases of acute myelocytic leukemia [41, 42] According to our experience, all cases of well differentiated monocytic leukemia



a, d *b e f* *c, g, h*

Fig 3 Comparison of the N AS esterase (a, d), the Sudan black B positivity (b, e, f) and the peroxidase activity (c, g h) of patient 1 (a, b, c) and patient 2 (d, e, f, g h) In figure 3b, c and g a weakly positive or negative leukemic monocytoid cell is shown near a moderately or strongly stained cell In figure 3e, a strongly and a weakly positive cell are compared to a strongly positive polymorphonuclear neutrophil (f) × 1,200

The index of the alkaline phosphatase in the mature granulocytes varied in each of the different patients and, allowing for the small number of cases studied, no precise statement can be made concerning this aspect No alkaline phosphatase was detected in the leukemic monocytoid cells [1, 36, 38] The results of the tests for the naphthylamidase presence varied from a low to a marked positive reaction in the smears tested When the PAS technique was applied, the resulting faint and diffuse stain could be prevented by saliva and 1,4-amyloglucosidase digestion, suggesting the presence of glycogen

Electron microscopical investigations (fig 5) The large atypical leukemic cells in the peripheral blood of both, patients 1 and 2, contained



Fig 4 Acid phosphatase Strong activity in the leukemic cells of patient 1 (a) and moderate and faint activity in the cells of patient 3 (b c, arrows) Nuclear stain Mayer s hemalum × 1 000

big lobulated nuclei with large nucleoli The nuclear chromatin was evenly distributed showing a condensed rim along the nuclear envelope Nuclear pockets were rarely observed Some finger-like cytoplasmic protrusions were distributed around the cell surface An abundance of free ribosomes was present in the cytoplasm as well as some rather long ergastoplasmic strands The Golgi apparatus appeared well developed and sometimes multipolar shaped Numerous, often very large mitochondria, distributed throughout the cytoplasm, contained dense matrices and large intramitochondrial granules Rare intracytoplasmic bundles of fibrils were observed, each fibril of which averaged about 100 A in diameter The plentiful small electron-dense granules (0 1 μm in diameter) were either uniformly distributed throughout the cytoplasm or arranged in groups Other, less electron-dense granules of major size resembled the typical 'primary' and rarely the 'secondary' granules of the neutrophil series Only in the leukemic cells of patient 2 a few Auer bodies were found No signs of phagocytosis occurred

Lysozyme For comparison the lysozyme levels of the urine and the serum of the 3 patients tested are shown in table II, along with the levels found in other types of leukemia In patients with acute myeloblastic leukemia the urinary lysozyme levels are usually low, whereas they may be high in some cases of acute myelocytic leukemia [41, 42] According to our experience, all cases of well differentiated monocytic leukemia

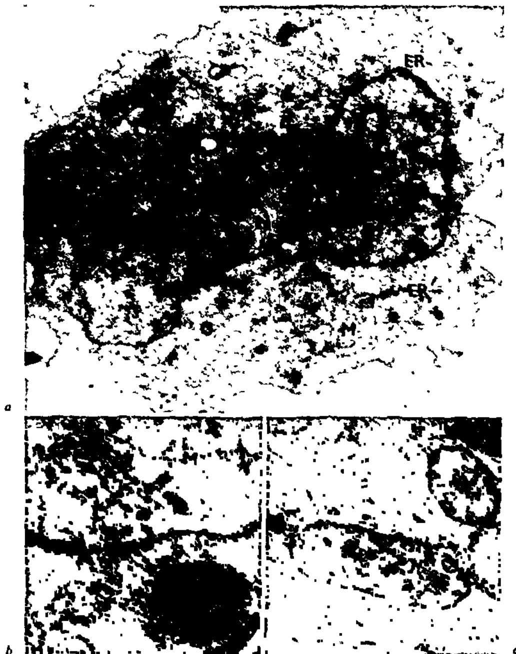


Fig. 5a. Leukemic cell of patient 1 showing a lobulated nucleus (N) with a large nucleolus (NL). The cytoplasm contain large ergastoplasmic profiles (ER), free ribosomes, a large Golgi apparatus (G), numerous mitochondria (M, see also in fig. 5c), and abundant granules (C, see also in fig. 5b). × 20,600. *b* Detail of figure 5a. Nucleus and nucleolus (NL). In the upper portion of the figure some small electron-dense granules are present measuring about 0.1 *µm* in diameter. × 47,000. *c* Detail of figure 5a. Mitochondrion with electron dense matrix and intramitochondrial granules (arrow). × 88,400.

Table II Urinary and serum lysozyme concentration and lysozyme excretion in some cases of monocytic and monomyelocytic leukemia

	Lysozyme concentration, μg/ml		Lysozyme excretion in urine in 24 h, mg
	serum	urine	
Normal range	5–10	0–3	0–4 5
Monomyelocytic leukemia			
Patient 1	–	1 5	2.4
Patient 2	40	300	360
Patient 3	63	500	650
Monocytic leukemia			
Patient 6	65	750	240
Patient 7	38	240	280

show prominent increases in both urinary and serum lysozyme levels [6, 7] Both, in these cases as well as in some myelocytic leukemia cases, most of the leukemic cells showed a strongly positive and specific labeling after the immunocytological demonstration of lysozyme [5, 7]

In contrast to the monocytic leukemia only 2 of the 3 studied 'monomyelocytic leukemia' cases showed great or extreme enzyme activities in both the serum and urine (patients 2 and 3) These higher enzyme levels were not associated with a more prominent monocyte-like cytochemical pattern as it would be expected (see fig 2 and table II) High enzyme levels have been observed in patient 3 in which the leukemic cells consisted of a rather poorly differentiated cell population In the 3 patients no relevant kidney dysfunction could be detected

Skin window investigations We previously reported on the high emigration rate of leukemic monocytes during the first hours of skin window experiments in patients with true monocytic leukemia [47, 50, 51] No such increased migration rate of the leukemic cells was noted in the 3 patients with 'monomyelocytic' leukemia in which this test had been performed Furthermore, the cells in these experiments failed to undergo extensive transformation into macrophages as manifested in both their morphological and cytochemical patterns In this respect, the 'monomyelocytic' leukemic cells behave differently from both normal and leukemic monocytes [47 51]

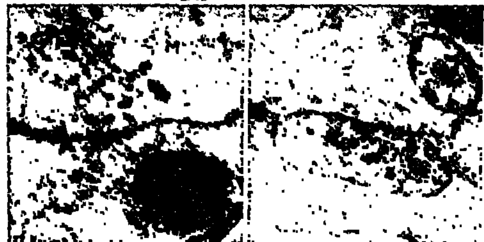


Fig 5a Leukemic cell of patient 1 showing a lobulated nucleus (N) with a large nucleolus (Nl) The cytoplasm contain large ergastoplasmic profiles (ER), free ribosomes, a large Golgi apparatus (G), numerous mitochondria (M, see also in fig 5c), and abundant granules (C, see also in fig 5b) × 20 600 *b* Detail of figure 5a Nucleus and nucleolus (Nl) In the upper portion of the figure some small electron-dense granules are present measuring about 0.1 μm in diameter × 47 000 *c* Detail of figure 5a Mitochondrion with electron dense matrix and intramitochondrial granules (arrow) × 88 400

that mature leukemic monocytes display and likewise also contained distinct immunocytologically demonstrable lysozyme protein The governing factors behind the high serum and urinary lysozyme concentrations are still poorly resolved problems Some of these factors may lie in the special nephrologic features working in combination with a rapid leukemic cell turnover and a possible enzyme leakage from the cells [39, 42, 60, 61] Due to the well-established fact that besides mature acute monocytic leukemia higher levels also occur in some cases of promyelocytic or myelocytic leukemia [6], and due to the questionable lysozyme turnover, some doubt has been raised as to whether the urinary and serum lysozyme concentration actually can be regarded as unique and distinguishing criteria when diagnosing monocytic leukemia [60, 61] Nevertheless, attention should be brought to the fact that the ultrastructur ally and cytochemically atypical 'monomyelocytic' leukemia cases may show a high intracellular lysozyme content as well as high serum and urinary levels [6, 52]

In skin window experiments the observed monomyelocytic cells behaved more like leukemic myeloblasts and promyelocytes, showing no tendency to migrate into the inflammatory areas, than like leukemic monocytes which abundantly migrate and subsequently transform into macrophages [47, 54]

The question now arises as to how such intermediate atypical cell forms may develop and in particular, in what ways they may differ from one another as far as concerns both the degree of maturation and the relative distribution of monocytic and promyelocytic features The explication of these parallel relationships and variations will be aided by two basical considerations The first concerns the origin of blood monocytes In contrast to SCHILLING s ancient opinion that monocytes would originate from the reticuloendothelial system, the myelogenous origin of the normal blood monocytes is now safely confirmed [15, 18, 30, 49, 51, 56, 59] Furthermore, cytochemical findings [9, 31, 52, 53] as well as results from specialized tissue culture investigations [37] suggest a close relationship among monocytes and neutrophil granulocytes, though the repeatedly stressed development of monocytes from mature promyelocytes containing an abundant equipment of cytochemically typical granules [9, 30, 31, 48, 49] deserves some doubt. Recent results indicate a less mature cell, for instance a myeloblast, or an earlier committed stem cell for both cell types as the linking progenitor cell [37, 53]

The second point concerns some suggestive recent data on the kinet

Discussion

The presented 4 deviating cases of myelogenous leukemia showed cytological features falling in between those typical of monocytic or of acute promyelocytic and myelocytic leukemia In the leukemic cells in question the intermediate position was manifested by the presence of distinct to prominent NaF-sensitive N-AS-esterase activity, otherwise detected only in true monocytic leukemia, and by similarly prominent positive peroxidase, N AS-Cl esterase, and Sudan black B staining reactions [31, 34, 47, 50, 53] When decisively positive, the latter combination provides the typical identifying guide lines of leukemias of the neutrophil granulocytic series [1, 19, 20, 53] Although the staining intensity of the NaF sensitive esterase reaction on the one, and Sudan black B and peroxidase staining on the other hand varied from case to case, it characteristically ranged between the patterns normally associated with monocytic leukemia at the one end and promyelocytic or myelocytic leukemia at the other (fig 2)

Ultrastructurally, the cells differed in such a manner from those of the normal cell series [4, 25, 57] and from the various types of acute leukemia observed until now, that they could not conclusively be said to belong to either group [4, 13, 17, 23, 24, 26] Some ultrastructural features, as for instance the copious free ribosomes, the long ergastoplasmic strands, the few granules closely resembling those of the neutrophils, the Auer bodies in one patient, and the large mitochondria, manifested the relationship of these particular cells to the neutrophil granulocytic series However, the presence of large lobulated nuclei and the preponderance in most of the leukemic cells, of small electron-dense granules, represented features ultrastructurally characterizing monocytic leukemia Hence the electron microscopical findings support the results of the cytochemical investigations both demonstrating characteristical features of promyelocytic and myelocytic as well as monocytic leukemias in these peculiar leukemic cell populations

Investigations of the urinary and serum lysozyme levels in 3 patients further underline the intermediate position of this leukemic cell population Two of the cases studied showed high lysozyme levels in all the available samples despite the fact that one of these cases was noted for rather poor cellular maturation [6, 47] Contrary to this, patient 1 had a relatively normal urinary lysozyme concentration, although his leukemic cells shared much the same cytochemical and ultrastructural features

that mature leukemic monocytes display and likewise also contained distinct immunocytologically demonstrable lysozyme protein The governing factors behind the high serum and urinary lysozyme concentrations are still poorly resolved problems Some of these factors may lie in the special nephrologic features working in combination with a rapid leukemic cell turnover and a possible enzyme leakage from the cells [39, 42, 60, 61] Due to the well-established fact that besides mature acute monocytic leukemia higher levels also occur in some cases of promyelocytic or myelocytic leukemia [6], and due to the questionable lysozyme turnover, some doubt has been raised as to whether the urinary and serum lysozyme concentration actually can be regarded as unique and distinguishing criteria when diagnosing monocytic leukemia [60, 61] Nevertheless, attention should be brought to the fact that the ultrastructurally and cytochemically atypical 'monomyelocytic' leukemia cases may show a high intracellular lysozyme content as well as high serum and urinary levels [6, 52]

In skin window experiments the observed monomyelocytic cells behaved more like leukemic myeloblasts and promyelocytes, showing no tendency to migrate into the inflammatory areas, than like leukemic monocytes which abundantly migrate and subsequently transform into macrophages [47, 54]

The question now arises as to how such intermediate atypical cell forms may develop and in particular, in what ways they may differ from one another as far as concerns both the degree of maturation and the relative distribution of monocytic and promyelocytic features The explication of these parallel relationships and variations will be aided by two basical considerations The first concerns the origin of blood monocytes In contrast to SCHILLING's ancient opinion that monocytes would originate from the reticuloendothelial system, the myelogenous origin of the normal blood monocytes is now safely confirmed [15, 18, 30, 49, 51, 56 59] Furthermore, cytochemical findings [9, 31, 52, 53] as well as results from specialized tissue culture investigations [37] suggest a close relationship among monocytes and neutrophil granulocytes, though the repeatedly stressed development of monocytes from mature promyelocytes containing an abundant equipment of cytochemically typical granules [9, 30, 31, 48, 49] deserves some doubt. Recent results indicate a less mature cell, for instance a myeloblast, or an earlier committed stem cell for both cell types as the linking progenitor cell [37, 53]

The second point concerns some suggestive recent data on the kinet-

ies and development of the leukemic processes [22, 29] This data, in addition to morphological and cytochemical findings, proposes new explanations of the immature or atypical cytological appearance of leukemic cell strains by suggesting their development from the pathological differentiation of immature cells [29, 43, 53], rather than from a tendency of already differentiated cells to lose their specific differentiation [31]

In the reported cases of myelogenous leukemia with 'monomyelocytic' cells, the pathological differentiation of the pathologic myeloid stem cells resulted in the simultaneous expression of both neutrophilic and monocytic features, as manifested by the production of monocytic and primary neutrophilic granules as well as of additional less typical cytological features in the same cells Only a few others, as far as we know, have reported observing similar atypical cell populations HERMANSKY, in 1968, made a reference to leukemic myelogenous cell populations presenting cytochemical patterns transitional between those of monocytes and neutrophils [20] LÖFFLER classified various types of acute leukemia into 5 cytochemically distinct groups [35], one of these, the 'peroxidase-esterase' type, could according to our opinion include cases similar to the 'monomyelocytic' type presented in this paper However, included in this 'peroxidase esterase' group may be mature promyelocytic leukemias which, as it has been frequently noted, may often display prominent NaF-resistant esterase activities [1, 20, 48, 53]

Repeated observations were made of leukemic processes, in which two or more cell lines simultaneously exhibited atypical features suggestive of some degree of leukemic degeneration Others reported cases in which the involvement of the erythropoietic and granulopoietic cell lines (erythroleukemia) has been morphologically manifested [1, 14, 35], and cases in which erythropoietic and monocytic [12], and granulopoietic and monocytic [16, 20, 35] cell lines were concerned BERNARD and SELIGMAN described the 'acute myelomonocytic leukemia of infants' as characteristically involving neutrophil, monocytic, and erythropoietic cell lines as well as immunoglobulin and hemoglobulin synthesis [11] Panmyelosis in adults, confirmed by cytochemical investigations, has been recently published [33] Summarizing, it should be pointed out that the 4 cases of 'monomyelocytic' leukemia presented in this paper are distinguished from the various other types of myelogenous leukemias which display mixed cell populations, in that their leukemic processes give rise to a single, fairly homogenous pathological cell population Similar atyp-

ical maturation has been noted in cases of myelogenous leukemia in which some cells exhibited typical features of both neutrophil and eosinophil granulocytes [46]

Due to the relative small number of cases available for examination, no clinical pattern particular to these peculiar monomyelocytic cases could be deduced with the exception that the patients were, as a rule, of an advanced age and that the fatal course was very rapid, though not as excessively rapid as frequently in promyelocytic leukemia Chemotherapeutic drugs could not be adequately tested and remissions lasting over a longer period of time could not be achieved

References

1 Abbrederis K Schmalzl F und Braunsteiner H Zur Differentialdiagnose akuter Leukamien mittels zytochemischer Methoden Schweiz. med Wschr *99* 1425 (1969)

2 Ackermann G A Histochemical demonstration of aminopeptidase activity in the leucocytes of blood and bone marrow J Histochem Cytochem *8* 386 (1960)

3 Ackermann G A Ultrastructure and cytochemistry of the developing neutrophil Lab Invest. *19* 290 (1968)

4 Anderson D R. Ultrastructure of normal and leukemic leucocytes in human peripheral blood J Ultrastruct Res Suppl *9* (1966)

5 Asamer H Schmalzl F und Braunsteiner H Der immunzytologische Lysozymnachweis in menschlichen Blutzellen. Acta haemat., Basel *41* 49 (1969)

6 Asamer H Schmalzl F und Braunsteiner H Die diagnostische und prognostische Bedeutung der Muramidase-(Lysozym) Bestimmung in Leukozytenlysaten Serum und Harn von Leukamiepatienten Klin Wschr *49* 587 (1971)

7 Asamer H Schmalzl F and Braunsteiner H Immunocytological demonstration of lysozyme (muramidase) in human leukemic cells Brit J Haemat *20* 571 (1971)

8 Bainton D F and Farquhar M G Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes II Cytochemistry and electron microscopy of bone marrow cells J Cell Biol *39* 299 (1968)

9 Bakalos D Monocytic precursors and their significance Atlas of bone marrow and lymph node cytology (Parissianos Athens 1965)

10 Barka, T and Anderson P J Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler J Histochem. Cytochem *10* 741 (1962)

11 Bernard J et Seligman M Les leucémies myélomonocytaires du nourrisson Nouv Rev franç Hémat *8* 759 (1968)

12 Broun G O Chronic erythromonocytic leukemia. Amer J Med. *47* 785 (1969)

13 CAPONE, R J Electron microscope studies on normal human myeloid elements Blood *23* 300 (1964)
14 DAMESHEK, W and GUNZ, F Leukemia (Grune & Stratton New York 1958)
15 EVERETT, N B and TYLER, R W Studies of lymphocytes relationships to mononuclear cells of inflammatory exudates Biochem Pharmacol Suppl (1968)
16 FISCHER, R Neue Aspekte der pathologischen Anatomie der Leukosen. Internist, Berl *9* 457 (1968)
17 FORTEZA BOVER, G, FORTEZA VILA, J, BAGUENA CANDELA, R., y PRIETO-GARCIA, F Ultrastructura de las cellulas de leucemias agudas humanas Sangre *12* 121 (1967)
18 FURTH R VAN and COHN, Z. A.. The origin and kinetics of mononuclear phagocytes J exp Med *128* 415 (1968)
19 HAYHOE, F G J, QUAGLINO, D., and DOLL, R Cytology and cytochemistry of acute leukemias (HMSO, London 1964)
20 HERMANSKY, F La valeur de quelque méthodes cytochimiques dans la classification des leucoses immatures et atypiques Nouv Rev franç Hémat. *8* 239 (1968)
21 HOTCHKISS, R. D A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations Arch Biochem *16* 131 (1948)
22 HUBER, C., HUBER H, SCHMALZL, F., and BRAUNSTEINER H Decreased proliferative activity of erythroblasts in granulocytic stem cell leukemia Nature Lond *229* 113 (1971)
23 HUHN, D und SCHMALZL, F Feinstruktur und Zytochemie der Paramyeloblasten Blut *23* 189 (1971)
24 HUHN D, SCHMALZL, F und DEMMLER, K Monozytenleukämie Licht und elektronenmikroskopische Morphologie und Zytochemie Dtsch med Wschr *96* 1594 (1971)
25 HUHN, D and STICH W Fine structure of blood and bone marrow An introduction to electron microscopic hematology (Lehmann München 1969)
26 ITO S Electron microscopic study of leukemic cells in the bone marrow Acta haemat jap *29* 22 (1966)
27 KAPLOW, L S A histochemical procedure for localizing and evaluating leukocyte alkaline phosphatase activity in smears of blood and bone marrow Blood *10* 1023 (1955)
28 KAPLOW, L S Simplified myeloperoxidase stain using benzidine dihydrochloride Blood 26 215 (1965)
29 KILLMANN, S A.. Acute leukemia development, remission relapse pattern, relationship between normal and leukemic hemopoiesis, and the 'Sleeper to-Feeder' stem cell hypothesis. Ser Haemat. *1* 103 (1968)
30 LEDER, L. D.. Fermentzytochemische Untersuchungen zur Herkunft des Blutmonozyten Klin. Wschr *44* 25 (1966)
31 LEDER L. D Der Blutmonozyt (Springer, Berlin 1967)
32 LÖFFLER, H Zytochemischer Nachweis der unspezifischen Esterase in Ausstrichen Klin Wschr *39* 1220 (1961)

33 LÖFFLER, H und LEDER L. D Panmyelose mit Panhämozytopenie Acta haemat., Basel *40* 75 (1968)

34 LÖFFLER, H Die Monozytenleukämie, in BRÜCHER Der Monozyt (Lehmann München 1969)

35 LÖFFLER H., Zytochemische Klassifizierung der akuten Leukosen, in STACHER Chemo- und Immunotherapie der Leukosen und malignen Lymphome (Bohmann, Wien 1969)

36 MELNICK, J P Histochemical enzymology of leukemic cells in AMROMIN Pathology of leukemia (Hoeber Medical Division, Harper & Row, New York 1968)

37 METCALF, D and MOORE, M A S Haematopoietic cells Their origin, migration and differentiation (Elsevier, Amsterdam 1971)

38 MOLONEY W C., MCPHERSON K., and FLIEGELMAN, L. Esterase activity in leukocytes demonstrated by the use of naphthol AS-D-chloracetate substrate J Histochem Cytochem *8* 200 (1960)

39 MUGGIA, F M, HEINEMANN, H O, FARHANGI, M., and OSSERMAN, E. F., Lysozymuria and renal tubular dysfunction in monocytic and myelomonocytic leukemia Amer J Med *47* 351 (1969)

40 NOVIKOFF, A. B Lysosomes in the physiology and pathology of cells contributions of staining methods, in Ciba Found Symp on 'Lysosomes' (Churchill, London 1963)

41 OSSERMAN, E F and LAWLOR D P Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J exp Med. *124* 921 (1966).

42 PERILLIE, P E, KAPLAN S S LEFKOWITZ, E. ROGAWAY, W., and FINCH, S. C. Studies of muramidase (lysozyme) in leukemia J amer med Ass. *203* 317 (1968)

43 PIERCE, G B Differentiation of normal and malignant cells Fed. Proc. *29* 1248 (1970)

44 REYNOLDS E S The use of lead citrate at high pH as an electron opaque stain in electron microscopy J Cell Biol *17* 208 (1963)

45 SCHAEFER, H E. und FISCHER, R. Der Peroxydasenachweis an Ausstrichpräparaten sowie an Gewebsschnitten nach Entkalkung und Paraffineinbettung. Klin. Wschr *46* 1228 (1968)

46 SCHAEFER H E., HELLRIEGL, K. P, HENNEKEUSER, H H., HÖRNER, G., ZACH, J und FISCHER, R. Unreifzellige myeloische Leukose mit chloracetatesterase positiver Eosinophilie Eosinophilenleukämie (in press)

47 SCHMALZL, F Unreifzellige Monozytenleukämie. Blut 22 157 (1971).

48 SCHMALZL, F und BRAUNSTEINER, H. Cytochemische Darstellung von Esteraseaktivitäten in Blut und Knochenmarkszellen. Klin. Wschr *46* 652 (1968)

49 SCHMALZL, F and BRAUNSTEINER, H On the origin of monocytes. Acta haemat., Basel *39* 177 (1968)

50 SCHMALZL, F und BRAUNSTEINER, H. Zur Zytochemie der Monozytenleukämie. Klin. Wschr *46* 1185 (1968)

51 SCHMALZL, F and BRAUNSTEINER, H Monocytes and macrophages – cytochemical investigations. Ser Haemat. *3(2)* 93 (1970)

13 CAPONE, R J Electron microscope studies on normal human myeloid elements Blood *23* 300 (1964)
14 DAMESHEK, W and GUNZ, F Leukemia (Grune & Stratton New York 1958)
15 EVERETT, N B and TYLER, R W Studies of lymphocytes relationships to mononuclear cells of inflammatory exudates. Biochem Pharmacol Suppl (1968)
16 FISCHER, R Neue Aspekte der pathologischen Anatomie der Leukosen Internist, Berl *9* 457 (1968)
17 FORTEZA BOVER, G, FORTEZA-VILA, J, BAGUENA CANDELA R., y PRIETO-GARCIA F Ultrastructura de las cellulas de leucemias agudas humanas. Sangre *12* 121 (1967)
18 FURTH R VAN and COHN, Z. A The origin and kinetics of mononuclear phagocytes. J exp Med *128* 415 (1968)
19 HAYHOE, F G J, QUAGLINO, D., and DOLL, R Cytology and cytochemistry of acute leukemias (HMSO, London 1964)
20 HERMANSKY, F La valeur de quelque méthodes cytochimiques dans la classification des leucoses immatures et atypiques Nouv Rev franç Hémat *8* 239 (1968)
21 HOTCHKISS, R D A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations Arch Biochem *16* 131 (1948)
22 HUBER, C., HUBER, H, SCHMALZL, F., and BRAUNSTEINER, H Decreased proliferative activity of erythroblasts in granulocytic stem cell leukemia Nature Lond *229* 113 (1971)
23 HUHN, D und SCHMALZL, F Feinstruktur und Zytochemie der Paramyeloblasten Blut *23* 189 (1971)
24 HUHN D, SCHMALZL, F und DEMMLER, K Monozytenleukämie. Licht und elektronenmikroskopische Morphologie und Zytochemie Dtsch med Wschr *96* 1594 (1971)
25 HUHN, D and STICH W Fine structure of blood and bone marrow An introduction to electron microscopic hematology (Lehmann, München 1969)
26 ITO S Electron microscopic study of leukemic cells in the bone marrow Acta haemat. jap *29* 22 (1966)
27 KAPLOW, L. S A histochemical procedure for localizing and evaluating leukocyte alkaline phosphatase activity in smears of blood and bone marrow Blood *10* 1023 (1955)
28 KAPLOW, L. S Simplified myeloperoxidase stain using benzidine dihydrochloride Blood *26* 215 (1965)
29 KILLMANN S A., Acute leukemia development, remission relapse pattern relationship between normal and leukemic hemopoiesis, and the 'Sleeper to-Feeder' stem cell hypothesis. Ser Haemat. *1* 103 (1968)
30 LEDER, L. D., Fermentzytochemische Untersuchungen zur Herkunft des Blutmonozyten Klin Wschr *44* 25 (1966)
31 LEDER, L. D Der Blutmonozyt (Springer, Berlin 1967)
32 LÖFFLER, H Zytochemischer Nachweis der unspezifischen Esterase in Ausstrichen Klin Wschr *39* 1220 (1961)

Acta Haemat. 48 89–97 (1972)

Actinomycin Binding Capacity in Human Leukaemic Lymphoid Cells

A. Pileri, P. Masera, N. Hulin and J. Brachet

Division of Haematology, Medical Clinic, University of Torino, Torino and Department of Molecular Biology, University of Bruxelles, Bruxelles

Abstract. Actinomycin binding which is considered as an expression of genetic activity, has been evaluated in acute and chronic lymphatic leukaemia cells and compared with values for both normal cells and pathologically (infectious mononucleosis) or PHA stimulated lymphocytes. A significant increase in binding accompanies lymphocyte activation. In acute leukaemia, the binding values were well above those observed in normal cells, and differences were noted between the proliferating and non proliferating blasts. In chronic lymphatic leukaemia, on the other hand, actinomycin binding is considerably decreased. This is taken as a sign of marked changes in genetic regulation.

Key Words
Actinomycin binding
Genetic regulation
Infectious mononucleosis
Lymphatic leukaemia
Lymphocyte stimulation
PHA stimulation

Various biochemical events connected with nucleic acid and protein metabolism in stimulated lymphocytes [1] have been examined in recent years. Blast transformation or activation is accompanied by increased labelled precursor incorporation into proteins and RNA, with predominant ribosomal RNA synthesis. This is followed by DNA replication usually followed by thymidine uptake [2–13].

Originally, the biochemical changes associated with such a 'stimulation' or 'transformation' were primarily explained as expressions of increased genetic activity, whereby a greater quantity of lymphocyte DNA would be made available for the transcription of messenger and ribosomal RNA [14]. The degree of uridine or other nucleoside incorporation, however, does not offer a direct measurement of RNA synthesis on account of the size and composition of the nucleotide pools to which the labelled nucleosides contribute [13]. Actinomycin binding to the chro-

52 SCHMALZL, F and BRAUNSTEINER, H Monomyelocytoid leukemia An unusual cytological type of myelogenous leukemia. Proc 5th Meet Europ Soc. Clin Invest., Scheveningen 1971

53 SCHMALZL, F and BRAUNSTEINER, H The application of cytochemical methods to the study of acute leukemia Acta haemat, Basel *45* 209 (1971)

54 SCHMALZL, F, HUBER H, ASAMER H, ABBREDERIS, K., and BRAUNSTEINER, H. Cytochemical and immunohistologic investigations on the source and the functional changes of mononuclear cells in skin window exudates. Blood *34* 129 (1969)

55 SHEEHAN H L and STOREY, G W An improved method of staining leukocyte granula with Sudan black B J Path Bact *59* 336 (1947)

56 SPECTOR W G, WALTERS N N, and WILLOUGHBY, D A The origin of mononuclear cells in inflammatory exudates induced by fibrinogen J Path Bact. *90* 181 (1965)

57 SPICER S S, HORN R. G., and WETZEL, B K Ultrastructural and cytochemical characteristics of leukocytes in various stages of development. Biochem Pharmacol *1968* 143

58 SPURLOCK P O, KATTINE, C. V., and FREEMAN, J A Technical modification in Maraglas embedding J Cell Biol *17* 203 (1963)

59 VOLKMAN, A and GOWANS J L The origin of macrophages from bone marrow in the rat Brit J exp Path *46* 62 (1965)

60 WIERNICK, P H and SERPICK, A A Clinical significance of serum and urinary muramidase activity in leukemic and other hematologic malignancies Amer J Med *46* 330 (1969)

61 YOUMAN J D, SAARNI M I., and LINMAN J W Diagnostic value of muramidase (lysozyme) in acute leukemia and proleukemia Mayo Clin Proc *45* 219 (1971)

Authors' addresses Dr F SCHMALZL, Dr ASAMER, Dr K ABBREDERIS and Dr H BRAUNSTEINER, Medizinische Universitätsklinik, *6020 Innsbruck* (Oesterreich), Dr D HUHN Institut für Hämatologie der Gesellschaft für Strahlen und Umweltforschung Assoziation mit Euratom Landwehrstrasse 61, *D-8 München 15* (Deutschland)

Acta Haemat. 48 89-97 (1972)

Actinomycin Binding Capacity in Human Leukaemic Lymphoid Cells

A PILERI P MASERA, N HULIN and J BRACHET

Division of Haematology Medical Clinic, University of Torino Torino and Department of Molecular Biology, University of Bruxelles, Bruxelles

Abstract Actinomycin binding, which is considered as an expression of genetic activity, has been evaluated in acute and chronic lymphatic leukaemia cells and compared with values for both normal cells and pathologically (infectious mononucleosis) or PHA-stimulated lymphocytes A significant increase in binding accompanies lymphocyte activation In acute leukaemia, the binding values were well above those observed in normal cells, and differences were noted between the proliferating and non proliferating blasts In chronic lymphatic leukaemia, on the other hand actinomycin binding is considerably decreased. This is taken as a sign of marked changes in genetic regulation

Key Words
Actinomycin binding
Genetic regulation
Infectious mononucleosis
Lymphatic leukaemia
Lymphocyte stimulation
PHA stimulation

Various biochemical events connected with nucleic acid and protein metabolism in stimulated lymphocytes [1] have been examined in recent years Blast transformation or activation is accompanied by increased labelled precursor incorporation into proteins and RNA, with predominant ribosomal RNA synthesis This is followed by DNA replication usually followed by thymidine uptake [2–13]

Originally, the biochemical changes associated with such a 'stimulation' or 'transformation' were primarily explained as expressions of increased genetic activity, whereby a greater quantity of lymphocyte DNA would be made available for the transcription of messenger and ribosomal RNA [14] The degree of uridine or other nucleoside incorporation, however, does not offer a direct measurement of RNA synthesis on account of the size and composition of the nucleotide pools to which the labelled nucleosides contribute [13] Actinomycin binding to the chro-

matin might give more direct information concerning genetic activity, since it seems to be related to the number of sites available to RNA polymerase for the purpose of genetic transcription [15–19] DARZYNKIEWICZ *et al* [19], for example, have noted increased chromatin binding of ³H actinomycin when lymphocytes are stimulated *in vitro* by phytohemagglutinins (PHA)

Abnormal patterns of lymphoid cell behaviour in acute and chronic lymphatic leukaemia might be attributable to disturbed genetic transcription and/or translation It was, therefore, decided to compare actinomycin binding in the leukaemic lymphoid cell *in situ* to that observed in both the normal cell and the pathologically (infectious mononucleosis) or PHA-stimulated cell

Material and Methods

Lymphoid cells from 9 patients were studied ³H actinomycin binding was autoradiographically studied with the technique of BRACHET and FICQ [20] in the following cells peripheral (cases 1 and 2) and marrow (case 1) lymphocytes from normal subjects lymphocytes stimulated with PHA at various times of culture (case 3) lymphomonocytes from a patient with infectious mononucleosis (case 4) at various stages of the disease peripheral and marrow lymphoid cells from patients with chronic (cases 5–7) and acute (cases 8 and 9) lymphatic leukaemia as well as lymph node lymphocytes in 2 of these cases (6 and 7)

Blood marrow and lymph node smears were fixed at 0 °C with 94% ethyl alcohol and acetic acid (70 30) and covered with 5 μCi/ml ³H labelled actinomycin (Schwartz, biologically prepared specific activity 6.5 Ci/mM) at room temperature for 1 h The slides were then treated with 5 μg/ml unlabelled actinomycin and washed in running water for 24 h They were then covered with emulsion exposed at 4 °C for 5 days developped fixed and stained with Unna Pappenheim or Giemsa.

The mean grain count (MGC) was determined on an average of 350 cells in each case The lymphocyte population was divided into small medium and large cells on the basis of nuclear diameter The same size criterion was applied to the acute leukaemia blasts.

All subjects in the series had received no treatment at the time of the experiment The leukaemia cases were in a steady state with more than 80% typical lymphoid cells in both the marrow and the circulating blood

Results

Acute lymphoblastic leukaemia (ALL) Actinomycin binding values for blasts were clearly higher than those observed in normal marrow or

Table I Acute lymphoblastic leukaemia. Marrow and peripheral blast actinomycin binding patterns in 3 cell classes with different nuclear diameter ranges (large>9 5 μm, medium=8–9 5 μm and small<8 μm

Cases	MGC (total population)	Large		Medium		Small	
		MGC	unlabelled cells, %	MGC	unlabelled cells, %	MGC	unlabelled cells, %
8, bone marrow	21 9	34 7	–	22.5	–	16 8	–
8, peripheral blood	20 3	24.2	–	20 5	–	17.2	–
9, bone marrow	24 9	32.3	–	25 1	–	20 0	–
9, peripheral blood	20 5	23 3	–	20.2	–	16.9	–

circulating lymphocytes (tables I and II) The large marrow blasts took up more of the labelled substance than their peripheral countreparts, although no statistically significant differences between the two compartments could be found One patient (case 8) presented about 10% lymphocytes in the circulating blood Their binding behaviour was similar to that observed in the normal subjects (table II)

Chronic lymphatic leukaemia (CLL) All 3 cases displayed a markedly decreased actinomycin binding by comparison with normal lymphocytes (table II), and many cells even proved incapable of any binding at all Binding values were depressed in both marrow and circulating cells, particularly in the latter Cells obtained from lymph nodes (cases 6 and 7) presented binding values that were not only higher than those observed in the marrow and circulating blood of the same subject, but were even higher than those found in marrow cells from normal subjects

Infectious mononucleosis (IM) Lymphomonocyte binding values were decidedly higher than those observed in the normal cell In the initial stages of the disease, they were even higher than those displayed by the lymphoblasts of acute leukaemia. Comparison of cells of similar nuclear size makes this increase quite clear (table I) Binding values fell to 50% of the initial levels 2 weeks after the onset of the disease, but they were still higher than those of the normal cells after more than 1 month after the onset of the disease (table III)

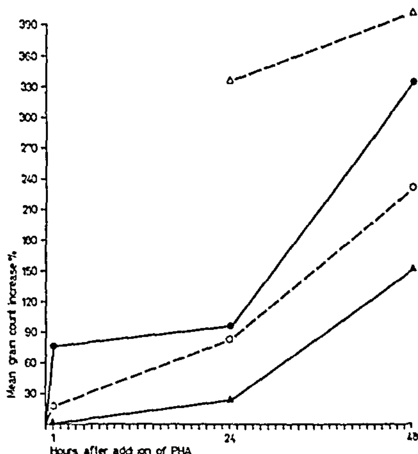


Fig 1 Actinomycin binding in PHA stimulated lymphocytes from a normal subject (case 3) Blast like (△) large (●) medium (○) and small (▲) lymphocytes

The data observed in patients with CLL are of particular interest, since the binding values were constantly depressed as compared to those observed in the normal subjects In fact, a certain number of cells are even incapable of binding any actinomycin This finding suggests severe genome repression and, in more general terms, a change in genetic regulation

This view is borne out by several cytochemical and biochemical findings For instance, the DNA histone ratio is changed due to increased histone levels [26] Since the DNA content remains normal [27], this is indicative of increased genetic repression in CLL lymphocytes Other biochemical findings give even more solid support to the suggestion of a disturbed genetic regulation in the CLL cell HENRY *et al* [28] have re-

ported a change in the sequential pattern of RNA synthesis, i e in the maturation of macromolecular (preribosomal) RNA into ribosomal RNA, in the chronic leukaemia leucocyte as compared to the normal one With RNA-DNA hybridization and hybridization-competition techniques, comparing rapidly labelled RNA from normal and CLL lymphocytes, NEIMAN and HENRY [29] demonstrated the presence in the diseased cell of RNA types that were absent or very rarely observed in the normal lymphocyte The ability to transport immunoglobulins to the cell surface, as in the case of the bone marrow derived lymphocyte (B lymphocyte), has also been recently reported in chronic leukaemia lymphocytes [30] Immunoglobulin values, however, are distinctly higher in the B lymphocyte from normal blood and it has, therefore, been suggested that CLL cells are grossly abnormal in this respect This is of course in good agreement with our finding of decreased actinomycin binding which would be an expression of altered genetic activity in these cells

Acknowledgements This investigation was supported by the Consiglio Nazionale delle Ricerche, Rome

References

1 NOWELL, P C Phytohemagglutinin as initiator of mitosis in cultures of normal lymphocytes Cancer Res *20* 462 (1960)
2 COOPER, H L and RUBIN A D RNA metabolism in lymphocytes stimulated by phytohemagglutinin Blood *25* 1014 (1965)
3 HAYHOE, F G J and QUAGLINO, P Autoradiographic investigations of RNA and DNA metabolism of human leucocytes cultured with phytohemagglutinin Uridine 5 ³H as a specific precursor of RNA Nature *205* 151 (1965)
4 KAY, J E RNA and protein synthesis in lymphocytes incubated with phytohemagglutinin, in The biological effects of phytohemagglutinin The Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, England pp 37–52 (1966)
5 COOPER H L Ribonucleic acid metabolism in lymphocytes stimulated with phytohemagglutinin II Rapidly synthesized ribonucleic acid and the production of ribosomal ribonucleic acid J biol Chem *243* 34 (1968)
6 BENDER, M A and PRESCOTT, D M DNA synthesis and mitosis of cultures of human peripheral leukocytes Exp Cell Res *27* 221 (1962)
7 MCINTYRE, O R and EBOUGH, F G The effect of phytohemagglutinin on leukocyte cultures as measured by P^{32} incorporation in the DNA, RNA and acid soluble fractions Blood *19* 443 (1962)

8 MAC KINNEY, A A STOHLMANN, F., jr., and BRECHER G The kinetics of cell proliferation in cultures of human peripheral blood Blood *19* 349 (1962)

9 RUBIN, A D and COOPER, H L Evolving patterns of R\A metabolism during transition from resting state to active growth in lymphocytes stimulated by phytohemagglutinin Proc nat Acad Sci, Wash *54* 469 (1965)

10 MUELLER, G C and LE MAHIEU, M Induction of ribonucleic acid synthesis in human leucocytes by phytohemagglutinin Biochim biophys Acta *114* 100 (1966)

11 PARENTI F, FRANCESCHINI P, FORTI G., and CEPPELLINI R. The effect of phytohemagglutinin on the metabolism and γ-globulin synthesis of human lymphocytes Biochim biophys Acta *123* 181 (1966)

12 POGO D G T, ALFREY, V G, and MIRSKY, A E RNA synthesis and histone acetylation during the course of gene activation in lymphocytes Proc nat Acad Sci, Wash *55* 805 (1966)

13 HAUSEN P and STEIN, H On the synthesis of RNA in lymphocytes stimulated by phytohemagglutinin Europ J Biochem *4* 401 (1968)

14 HIRSCHHORN, R, TROLL, W, BRITTINGER, G, and WEISSMANN G Template activity of nuclei from stimulated lymphocytes Nature *222* 1247 (1969)

15 BRACHET, J and HULIN, N Binding of tritiated actinomycin and cell d fferentiation Nature *222* 481 (1969)

16 DARZYNKIEWICZ, Z., GLEDHILL, B L., and RINGERTZ, N R Changes in deoxyribonucleoprotein during spermiogenesis in the bull [3]H actinomycin binding capacity Exp Cell Res *58* 435 (1969)

17 BERLOWITZ, L., PALLOTTA D J., and SIBLEY, C H J Chromatin and histones Binding of tritiated actinomycin D to heterochromatin in mealy bugs Science *164* 1527 (1969)

18 BRACHET, J and HULIN N Actinomycin binding in differentiating and dividing cells Exp Cell Res *59* 486 (1970)

19 DARZYNKIEWICZ, Z., BOLUND L., and RINGERTZ, N R Actinomycin binding of normal and phytohaemagglutinin stimulated lymphocytes. Exp Cell Res *55* 120 (1969)

20 BRACHET, J and FICQ A Binding sites of [14]C actinomycin in amphibian oocytes and an autoradiography technique for the detection of cytoplasmic DNA Exp Cell Res *38* 153 (1965)

21 *GAVOSTO F, PILERI A., and MARAINI G Incorporation of thymidine* labeled with tritium by circulating cells of infectious mononucleosis Nature *183* 1691 (1959)

22 GAVOSTO F PILERI, A BACHI, C., and PEGORARO L Proliferation and maturation defect in acute leukaemia cells Nature *203* 92 (1964)

23 GAVOSTO F, PILERI A, GABUTTI V., and MASERA P Cell population kinetics in human acute leukaemia. Europ J Cancer *3* 301 (1967)

24 PILERI, A, TAROCCO R P, GAVOSTO F, PONZONE, A e NICOLA P Caratteristiche della proliferazione cellulare nelle leucemie linfoblastiche infantili Tumori *54* 147 (1968)

25 PILERI A PONZONE, A TAROCCO R P., and GAVOSTO F Unresponsiveness

to phytohaemagglutinin of acute lymphoblastic leukaemia cells Lancet i 409 (1968)

26 PERUGINI S, TORELLI, U., and SOLDATI, M Differences in the desoxyribonucleoprotein complex of normal and leukemic human lymphocytes. Experientia 13 441 (1957)

27 BECK, W S Leukocyte metabolism in JENSEN and KILLMANN Leukocytes, eosinophils, basophils, biochemistry, pp 69–93 (1968)

28 HENRY, P H, REICH P, KARON M., and WEISMAN, S Characteristics of RNA synthesized in vitro by lymphocytes of chronic lymphocytic leukemia J Lab clin Med 69 47 (1967)

29 NEIMAN P E and HENRY, P H Ribonucleic acid-deoxyribonucleic acid hybridization and hybridization-competition in studies of the rapidly labeled ribonucleic acid from normal and chronic lymphocytic leukemia lymphocytes. Biochemistry 8 278 (1969)

30 WILSON J D and NOSSAL, G J V The T or B cell nature of chronic lymphocytic leukaemic lymphocytes Lancet ii 788 (1971)

Authors addresses A PILERI and P MASERA, Divison of Haematology, General Clinic University of Torino, Corso Polonia 14, 10126 Torino (Italy) J BRACHET and N HULIN Department of Biology University of Bruxelles, Rue des Chevaux 67 Rhode St Genese Bruxelles (Belgium)

Acta Haemat 48 98–103 (1972)

Familial Leukaemia

M L Ghosh

Department of Haematology District General Hospital Barnsley Yorkshire

Abstract Two instances of leukaemia occurring in siblings among 31 cases in a 3 year period study in a densely populated medium sized town and surrounding areas with an approximate population of 200 000 are reported The incidence of familial leukaemia is unusually high (6%) although the general incidence remains approximately the same when compared with regional and national figures Multiple cases of leukaemia and cancer among the members of the families in this report are thought to be influenced by hereditary factors Chromosome analysis in two siblings revealed polyploid abnormality in 95% of bone marrow cells in one case

Key Words
Familial leukaemia
Heredity in leukaemia
Leukaemia and cancer

Although leukaemia in siblings is rare, yet multiple cases of leukaemia among siblings and/or relatives of a family have been reported by various authors [1, 4, 7, 11, 12, 15, 16] Wintrobe [17] recorded 65 cases of familial leukaemia There is difference of opinion regarding the actual incidence of familial leukaemia as most of the reports consist of one or two families except those of retrospective analysis In view of the rarity of the condition it is worth reporting 4 cases of leukaemia in siblings found in a relatively small population The interesting feature in this report is the occurrence of identical type of leukaemia and or cancer among the siblings at approximately the same age brings up the possibility of genetic influence in these particular families

Patients and Methods

Routine haematological investigations were carried out on all patients referred to hospital (serving a population of approximately 200 000) since October 1968 for a period of 3 years. Detailed clinical examinations were made on suspected

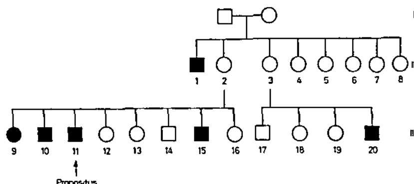


Fig 1 The B family pedigree ■ = Affected male, ● = affected female

cases and their past and family history carefully recorded The diagnosis was es tablished with repeated blood and bone marrow examinations including cytochemi cal stain and chromosome studies where indicated Subsequent course was followed up by blood and bone marrow examinations at regular intervals

The 'B Family (fig 1)

Case 1 E B, propositus (III_{11}), a male aged 41 years was admitted to hospi tal in April, 1970 with history of tiredness cervical lymphadenopathy for 2 months and bruising for 1 month There is no consanguinity in the family One sister (III_9) died of carcinoma of the stomach at the age of 23 years One brother (III_{10}) died of reticulosarcoma of the lung at the age of 33 years A maternal un cle (II_1) died of bronchogenic carcinoma and a cousin (III_{20}) died of acute lym phoblastic leukaemia

He was very pale with extensive purpura and ecchymoses and generalised lym phadenopathy The liver was enlarged 4 cm and the spleen 2 cm Hb 6 8 g/100 ml, platelets 30 000/mm³, WBC 148,000/mm³, myelomonoblasts 44%, neutrophils 15%, eosinophils 8% lymphocytes 16%, monocytes 10%, promonocytes 7% Bone marrow aspiration showed myelomonoblasts 58% and many immature and mature monocytes confirming acute myelomonocytic leukaemia He was treated with prednisolone, 6-mercaptopurine and fresh blood transfusion but he died 3 days later of cerebral haemorrhage Post mortem examination confirmed extensive leu kaemic infiltration of many viscera, skin and lymph nodes and cerebral haemorrhage

Case 2 C B (III_{15}) a male aged 31 years, a brother of the above patient, was admitted to hospital in July, 1971, with history of fainting, tiredness, sweating and weakness for 3 weeks following a dental extraction and gum bleeding lasting for 24 h He was severely anaemic and had many enlarged cervical glands with some purpuric spots over the body The liver was enlarged 1 cm, the spleen was not palpable Hb 4 7 g/100 ml, platelets 25,000/mm³, WBC 5,000 mm³, myelomonob-

lasts 12% neutrophils 40% lymphocytes 33% monocytes 15% Bone marrow aspiration showed an identical picture to that of his brother (case 1) with myelomonoblasts 70% increased promonocytes and monocytes confirming acute myelomonocytic leukaemia He was treated with prednisolone 6-mercaptopurine and methotrexate and fresh blood transfusion but with no response and he died 4 weeks later Post mortem was not permitted

The M Family

Case 3 A M, a 17 year old girl was admitted to hospital in February, 1971 with 4 weeks history of generalised musculo skeletal pain, vomiting and weight loss No history of consanguinity A maternal uncle died of carcinoma of the prostate

She was extremely anaemic and there were a few small cervical lymph nodes The liver was enlarged 4 cm the spleen 3 cm Hb 4.5 g/100 ml platelets 28 000 mm³ WBC 6 500 mm³, neutrophils 20% lymphocytes 35% blast cells 45% with predominantly lymphoblasts Bone marrow aspiration showed 60% blast cells composed mainly of lymphoblasts and some undifferentiated stem cells and considered to be acute lymphoblastic leukaemia with strong PAS positivity by cytochemical study Repeated chromosome analysis of bone marrow aspiration by the method of TJIO and WHANG [14] did not show any abnormality She was treated with fresh blood transfusion prednisolone 6-mercaptopurine with complete remission confirmed by blood and bone marrow examination Remission was maintained with 6-mercaptopurine for 11 months when she relapsed with increased blast cells in the peripheral blood and bone marrow She was treated with prednisolone and vincristine and complete remission was achieved and maintained at present with intermittent parenteral methotrexate as oral therapy caused severe diarrhoea and vomiting

Case 4 D C.M., an 8 year old boy brother of the above patient (A M) was admitted to hospital in June 1971 with 1 month s history of tiredness lethargy and anorexia He looked thin very pale with many petechial spots and generalised lymphadenopathy The liver was enlarged 4 cm the spleen 5 cm Hb 6 6 g 100 ml platelets 25 000 mm³, WBC 8 000 mm³, neutrophils 15% lymphocytes 55% blast cells 30% mainly lymphoblasts and undifferentiated blast cells Bone marrow aspiration showed approximately 80% blast cells consisting predominantly of lymphoblasts and some stem cells as well Cytochemical stain showed increased PAS activity confirming acute lymphoblastic leukaemia similar to that of his sister (A M) Chromosome analyses of direct bone marrow preparation showed 95% of the cells were polyploid but skin fibroblast culture showed no chromosome abnormality He was treated with platelet rich blood prednisolone vincristine but he died 2 weeks later of cerebral haemorrhage Post mortem was not permitted

Results

Table I gives the types of leukaemia and age distribution of the patients diagnosed A total of 31 cases of leukaemia were diagnosed during

Table I Types of leukaemia and age distribution

	Age group, years			
	0–4	5–20	21–60	>61
Acute lymphoblastic	2	3	1	0
Acute myeloblastic	2	2	3	2
Acute myelomonocytic	0	0	3	1
Chronic myeloid	0	0	2	1
Chronic lymphatic	0	0	2	6
Di-Guglielmo's disease	0	0	1	0
Total	4	5	12	10

a 3-year period study since October, 1968, with an incidence of 5.2 per 100,000 per annum. This is similar to that of Sheffield registration rate in 1963–1966 of 5.9 for males and 5.2 for females [5], but higher in comparison with the death rate for England and Wales in 1966, when the figures were 3.4 and 2.5, respectively [10]. Amongst the 31 patients, 20 and 11 cases were of acute and chronic leukaemia, respectively, and 2 instances of the disease (6%) occurred in siblings – unduly high incidence than expected.

Discussion

Although the exact incidence of familial leukaemia is not known, the instances are too high to be regarded as chance occurrence. VIDEBAEK [15] studied the pedigree of 209 patients with leukaemia and found 17 (8%) had one or more relatives who had suffered from leukaemia compared with 0.5% among 200 control families and he suggested hereditary influence of dominant character is an important factor in some cases. In a recent study, VIDEBAEK [16] reported a further 3 cases of familial leukaemia occurring within 1 year. High incidence of familial leukaemia was denied by others [6, 8, 9, 12], however, STEINBERG [12] accepted he hereditary influence in certain families. ANDERSON [1] reported such ι family in which 5 of the 8 children died of lymphatic leukaemia. According to GUASCH [6], 1 in 220 cases of leukaemia could be expected o be familial. BRIDGES and NELSON [3] found 3 instances of leukaemia occurring more than once in a family among 182 cases. BARBER and

SPIERS [2] found 8 affected siblings among 1,795 leukaemic children instead of the expected 1.5 and the present report confirms the increased incidence of leukaemia in siblings.

Significantly increased incidence of cancer was found among the relatives of leukaemic patients [9, 16]. STEINBERG [12] described a family in which 3 out of the 7 siblings died from acute leukaemia and the fourth from lymphosarcoma. STEWART [13] found increased incidence of leukaemia and cancer among the siblings in a study of 1,638 children dying of leukaemia or cancer. SNYDER *et al.* [11] saw 6 cases of acute myeloid leukaemia including 3 siblings and 2 cases of reticuloendotheliosis in a family. The 'B' Family in this present report is of particular interest in that the occurrence of multiple cases of carcinoma and leukaemia among the siblings and the maternal relatives of the propositus (III_{11}) suggest the presence of a genetic influence of a dominant character in that branch of the family.

Chromosome studies have so far been unsuccessful in determining the hereditary influence of familial leukaemia as deletion and polyploid abnormality described in such cases are inconstant and non-specific. In this present study direct bone marrow preparation of the patient (case 4) showed 95% of the cells were polyploid, whereas his sister (case 3) did not show any chromosome abnormality. The polyploid abnormality of the marrow cells associated with normal chromosome constitution of skin fibroblast culture suggests a malignant nature of the haemopoietic cells due to somatic mutation rather than inherent chromosome anomaly. Such an anomaly is unlikely to be a basic feature in familial leukaemia but genetic abnormalities, many of which are not recognisable cytologically at present, do not rule out the hereditary influence, at least in some cases, of familial leukaemia.

References

1 ANDERSON, R. C.: Familial leukemia. A report of leukemia in five siblings with a brief review of the genetic aspects of this disease. Am. J. Dis. Child. *81:* 313–322 (1951).

2 BARBER, R. and SPIERS, P.: Oxford survey of childhood cancer. Progress Report II. Mthl Bull. Minist. Hlth *23:* 46–52 (1964).

3 BRIDGES, J. M. and NELSON, M. G.: Familial leukaemia. Acta haemat., Basel *26:* 246–251 (1961).

4 CAMPBELL, W. A. B.; MACAFEE, A. L. and WADE, W. G.: Familial neonatal leukaemia. Arch. Dis. Childh. *37:* 93–98 (1962).

5 Cancer Registration in Sheffield Region 1963–1966 (Sheffield Regional Hospital Board Sheffield 1969)
6 GUASCH J Hérédité des leucémies Sang 25 384–421 (1954)
7 GUNZ, F W, FITZGERALD P H, CROSSEN P E, MACKENZIE, I S, POWLES C. P., and JENSEN, G R Multiple cases of leukaemia in sibship Blood 27 482–489 (1966)
8 KALIAMPETSOS G Kommen Blutkrankheiten und Karzinome unter den Verwandten von Leukamiekranken gehäuft vor? Dtsch med Wschr 79 1783–1785 (1954)
9 MORGANTI G et CRESSERI, A Nouvelles recherches genetiques sur les leucémies Sang 25 421–453 (1954)
10 The Registrar General s Statistical Review for England and Wales, 1966 (HMI Stationery Office London 1967)
11 SNYDER A. L. LI F P, HENDERSON, E S., and TODARO G J Possible inherited leukaemogenic factors in familial acute myelogenous leukaemia Lancet i 586–589 (1970)
12 STEINBERG A G The genetics of acute leukemia in children Cancer Philad 13 985–999 (1960)
13 STEWART A Aetiology of childhood malignancies Congenitally determined leukaemias Brit med J i 452–460 (1961)
14 TJIO, J H and WHANG J Chromosome preparations of bone marrow cells without prior in vitro culture or in vivo colchicine administration Stain Technol 37 17 20 (1962)
15 VIDEBAEK A Heredity in human leukaemia and its relation to cancer (Lewis London 1947)
16 VIDEBAEK A Familial leukaemia Acta path microbiol scand 44 372–376 (1958)
17 WINTROBE M M Clinical haematology (Lea & Febiger Philadelphia 1967)

Author s address Dr M L GHOSH Department of Haematology District General Hosp tal Barnsley Yorks (England)

Nukleoli unterscheiden liessen Er verwendete eine nach STOCKINGER und KELLNER [26] modifizierte Methylenblaufarbung die auf Arbeiten von PISCHINGER [23] zuruckgeht Die Methode beruht auf der unter schiedlichen Anfarbbarkeit verschiedener Zellstrukturen in Abhangigkeit vom isoelektrischen Punkt So konnen bei pH 4–5 die Ribonukleinsau ren in Nukleolus und Zytoplasma dargestellt werden wahrend die Des oxyribonukleinsauren des Kernes ungefarbt bleiben Daraus ergibt sich eine fast selektive Anfarbbarkeit der Nukleolen in Lymphozytenkernen Ein weiteres Verdienst gebuhrt der Schule von PLENERT [24] die 1966 die Methode im wesentlichen dadurch verbesserte, dass sie als Farbstoff Toluidinblau verwendete

Grosse und Haufigke t der Nukleolen konnen unter bestimmten Bedingungen die Aktivitat des Zellstoffwechsels widerspiegeln [7] es wird auch diskutiert dass d e Art von Nukleolenbild und bildung nicht zufallig und allgemein aufs engste mit den chromosomalen Gegebenheiten verknupft ist [1 2]

In den letzten Jahren sind eine Reihe von Studien bekannt geworden, die versuchten die Besonderheiten des Nukleolenbildes von Lymphozytenpopulationen unter verschiedenen Bedingungen auszudeuten An ande rer Stelle ist eine ausfuhrliche Darstellung gegeben [10] Sie soll hier eingangs als Überblick erfolgen um die Problematik verstandlich zu machen Die vorliegende Arbeit hatte die Aufgabe, unter Berucksichti gung bekannter Charakteristika das Nukleolenbild von Lymphozytenpo pulationen aus dem peripheren Blut mittels der Toluidinblaufarbung darzustellen und anhand folgender Vergleichsgruppen zu analysieren ge sunde Personen, desgleichen Kinder mit bakteriellen Infektionen (anti biotische Behandlung mit bzw ohne Gabe von Corticosteroiden) ferner unter langerer Behandlung mit Corticosteroiden sowie Zytostatika

Literatur

GRUNDMANN [11] beschrieb 1957 an jungen Ratten in Milz und Lymphknoten nach Lokalisation und Morphologie zwei Entwicklungsreihen der Lymphopoese d e in je 6 Stufen von einer hellkernigen undifferenzierten Retikulumzelle zu rei fen Lymphozyten fuhren Die reifen Stufen in den Follikeln besassen einen gros sen zentralstandigen Nukleolus, die in den Sinus der Lymphknoten gelegene meh rere kleine nukleolenartige Verdichtungen Der Autor sprach demgemass von Fol likel und Sinuslymphozyten Beide Zelltypen konnten mit verschiedenen histologi schen Techniken in allen Reifungsstufen voneinander unterschieden werden und waren auch im peripheren Blut erkennbar Seine we teren Untersuchungen ergaben

Acta Haemat. 48: 104–115 (1972)

Die Nukleolen-Morphologie von Lymphozyten des peripheren Blutes[1]

E. AMBS und R. GRONEMEYER

Universitäts-Kinderklinik (Direktor: Prof. Dr. J. STRÖDER), Würzburg

Abstract. Nucleoli of lymphocytes from peripheral blood can be demonstrated by methylene blue staining according to the method of PISCHINGER modified by STOCKINGER and KELLNER. This allows their classification in macro and polynucleolar lymphocytes. The application of toluidine blue staining according to PLENERT made it possible to differentiate peripheral lymphocyte populations of children without any haematological diseases treated for longer periods with corticosteroids or cytostatic drugs (immunosuppressive therapy). Compared with control groups (normal persons, children with bacterial infections treated with antibiotics, partly for a short period with corticosteroids) the percentage of macronucleolar lymphocytes increased while polynucleolar types were diminishing. An interpretation is not yet possible.

Key Words
Agammaglobulinaemia
Corticosteroid therapy
Immunosuppressive therapy
Lymphocyte nucleoli
Toluidine blue

Die Fortschritte der Immunologie haben eine zunehmende Aufklärung der Lymphozytenfunktion gebracht [9]. Dabei war es möglich, mehrere Gruppen innerhalb der Zellen mit lymphozytärer Morpholo[gie] zu charakterisieren, was morphologischen Studien sowie zytochemi[schen] wie histochemischen Untersuchungen bis dahin nicht befriedigen[d mög]lich gewesen war [3]. Die Morphologie der Lymphozytenpop[ulation im] peripheren Blut zeigt eine gewisse Uniformität, auffallend[e Charakteri]stika einzelner Lymphozyten weisen fliessende Übergän[ge innerhalb der] Population auf, so dass Abgrenzungen schwierig sind.

Im Jahre 1957 publizierte GRUNDMANN [11] ei[ne ...] der sich Lymphozyten nach den morphologisch[en ...]

[1] Herrn Prof. Dr. J. STRÖDER, Direktor der U[niversitäts-Kinderklinik Würz]burg, zum 60. Geburtstag gewidmet.

Zusammenfassend bestehen in der Verteilung der makro- und polynukleolären Lymphozyten zwischen den Gruppen A, B und C einerseits und den Gruppen D und E andererseits hochsignifikante Unterschiede, wobei bei Patienten, die unter einer längeren Therapie mit Prednison oder dem Endoxanderivat ASTA Z 4942 standen, der Anteil der Makronukleolaren zuungunsten der Polynukleolaren zunahm Das gilt fur den Vergleich mit der Gruppe gesunder, junger Erwachsener Im Vergleich mit diesen zeigen Kinder mit bakteriellen Infektionen mit oder ohne kurzzeitige Prednisontherapie keine signifikanten Abweichungen Andererseits können im Varianzvergleich Unterschiede zwischen der Kontrollgruppe und der mit bakteriellen Infekten, insbesondere der Prednisongruppe, statistisch gesichert werden

Erganzend soll noch erwahnt werden, dass die beiden Kinder mit Antikörpermangelsyndrom relativ hohe Werte an Makronukleolaren erreichten (94 bzw 97,5%) Lymphoidzellen und nichtdifferenzierbare mononukleäre Zellen weisen im Vergleich zu beiden Lymphozytengruppen keine auffälligen Veranderungen auf

Diskussion

Bei den Kindern, die über langere Zeit mit Corticosteroiden bzw einem Zytostatikum behandelt wurden, lasst sich eine Verschiebung des Anteils der makro- und polynukleolären Lymphozyten derart erkennen, dass der prozentuale Anteil der Makronukleolaren ansteigt Dies ist nicht aus der Art der morphologischen Klassifizierung zu deuten Ob und in welchem Zusammenhang diese Veranderungen mit funktionellen Vorgängen stehen, lasst sich nicht sagen

Lediglich erwahnt sollen die Befunde an beiden Kindern mit Agammaglobulinamien werden, wobei aber keinerlei Aussagen moglich sind wahrend hier bei einer kongenitalen Agammaglobulinamie, Typ Bruton, und einer erworbenen Verminderung von IgG eine relative Zunahme der Makronukleolaren *beobachtet wurde, konnte* GRUNDMANN *[16]* bei einem Kind mit einer schweren Lymphopenie das völlige Fehlen der (makronukleolaren) Follikellymphozyten feststellen, offensichtlich handelte es sich hierbei um die «Schweizer Form» der Agammaglobulinamie

Zusammenfassung

Die von STOCKINGER und KELLNER modifizierte Methylenblaufärbung nach PISCHINGER erlaubt, Nukleolen von Lymphozyten aus dem peripheren Blut darzustellen, wodurch eine Klassifizierung in makro- und polynukleoläre Lymphozyten möglich wird Bei Anwendung der Toluidinblaumodifikation nach PLENERT war es möglich Unterschiede in der Zusammensetzung peripherer Lymphozytenpopulationen bei Kindern ohne Erkrankungen des blutbildenden Organs zu finden, die längere Zeit mit Corticosteroiden oder zytostatisch (immundepressiv) behandelt wurden Im Vergleich zu Kontrollgruppen (Normale, Kinder mit bakteriellen Infektionen und antibiotischer Therapie mit und ohne kurzzeitige Corticosteroidgabe) nahm der Prozentsatz der makronukleolären zuungunsten der polynukleolaren Lymphozyten zu. Diese Verschiebungen sind statistisch signifikant Eine Interpretation aufgrund der erhaltenen Ergebnisse ist noch nicht möglich

Für die Beratung und Durchführung bei der statistischen Auswertung sind wir Herrn Dipl Mathematiker Dr BERND KNAUER vom Rechenzentrum der Universität Würzburg (Institut für Angewandte Mathematik, Vorstände Prof Dr J STOER und Prof Dr W VELTE), für die Untersuchungen der Immunglobuline im Serum Herrn Priv Doz. Dr C. MIETENS, Universitäts Kinderklinik Würzburg, zu grossem Dank verpflichtet

Literatur

1 ALTMANN, H W Allgemeine Morphologie der Zelle, in Hb ges Hämatol., vol I/1, p 85 (Urban & Schwarzenberg München)

2 ALTMANN, H -W und GRUNDMANN, E Phasenkontrastoptische Untersuchungen zur Vitalstruktur tierischer Zellkerne Beitr path Anat *115* 313 (1955)

3 AMBS, E. Zur Klassifizierung von Lymphocyten und Lymphoidzellen im peripheren Blutbild Ann paediat., Basel *203* 215 (1964)

4 AMBS E. und HILD K B Die Blutbildveränderungen des Pfeifferschen Drüsenfiebers in Abhängigkeit vom Krankheitsstadium. Arch Kinderheilk *181* 236 (1970)

5 AMBS, E. und SALÁT, W Differenzierungsversuch von Lymphocyten aus dem Blut von Kindern im ersten Lebensjahr nach dem Nukleolenbild Arch. Kinderheilk. *172* 324 (1965)

6 BEGEMANN, H, RASTETTER H und FINK, U Zur Klassifizierung der Lymphocyten Med Klin *58* 706 (1963)

7 CASPERSSON T., Cell growth and function (Norton, New York 1950)

8 CAVALLI-SFORZA L. Biometrie Grundzüge biologisch medizinischer Statistik, 2. Aufl (Fischer, Stuttgart 1969)

9 CRADDOCK, C. G, LONGMIRE, R., and MCMILLAN, R. Lymphocytes and the immune response New Engl J Med *285* 324 378 (1971)

10 GRONEMEYER, R. Das Nukleolenbild von Lymphocyten aus dem peripheren Blut unter verschiedenen Bedingungen, Med Inaug Diss., Würzburg (im Druck)

11 GRUNDMANN, E. Cytologische Untersuchungen über Formen und Ort der Lymphocytenreifung bei der Ratte Verh dtsch Ges Path *41* 261 (1957)
12 GRUNDMANN, E Experimentelle Untersuchungen über die funktionelle Cytomorphologie der lymphatischen Strukturen unter dem Einfluss von Cortison und DOCA. Beitr path. Anat. *119* 377 (1958)
13 GRUNDMANN, E. Lymphocyten und Plasmazellen im lymphatischen Gewebe der Ratte Beitr path Anat. *119* 217 (1958)
14 GRUNDMANN E Über die Unterscheidung von zwei Lymphocyten Typen im Phasenkontrastmikroskop Virchow s Arch. path Anat *332* 17 (1959)
15 GRUNDMANN, E Weitere Untersuchungen über die Lymphocytenbildung Verh dtsch Ges Path. *42* 211 (1959)
16 GRUNDMANN, E. Der morphologische Nachweis von zwei Lymphocytensystemen beim Menschen Klin. Wschr *37* 941 (1959)
17 GRUNDMANN, E Neuere Befunde über Entstehung und Bedeutung der Lymphocyten Dtsch med. Wschr *85* 741 (1960)
18 JORKE, D und WEDEKIND, U L. Das Verhalten verschiedener Lymphocytenformen und Lymphoidzellen unter dem Einfluss ionisierender Strahlen. Fortschr Röntgenstr *108* 104 (1968)
19 KEHNSCHERPER, M Leukocytenstudien im Kindesalter Habil-Schrift Jena (1969)
20 KEHNSCHERPER, M und KUSAK, H Das Verhalten der Lymphocyten bei Frühgeborenen Folia haemat., Lpz *87* 218 (1967)
21 KOLLER, S Neue graphische Tafeln zur Beurteilung statistischer Zahlen (Steinkopff Darmstadt 1969)
22 MICHALOWSKI, H und PLENERT, W Studien zur Differenzierung der Lymphocyten des peripheren Blutes im Kindesalter II Bakterielle und Virus-Infektionen Folia haemat., Lpz. *85* 227 (1966)
23 PISCHINGER A. Die Lage des isoelektrischen Punktes histologischer Elemente als Ursache ihrer verschiedenen Farbbarkeit Z. Zellforsch *3* 169 (1926)
24 PLENERT, W, MICHALOWSKI, H und KEHNSCHERPER M Studien zur Differenzierung des peripheren Blutes im Kindesalter I Methodik und Normalwerte in verschiedenen Altersgruppen. Folia haemat., Lpz. *85* 218 (1966)
25 STACHER, A Zur Morphologie der Blutlymphocyten nach Pyrogen und ACTH. Wien Z. inn Med *44* 110 (1963)
26 STOCKINGER, L. und KELLNER, G Der Lymphocytennukleolus Wien. Z. inn Med *33* 135 (1952)
27 ZOGRAPHOV, D G und GOSPODINOWA, D N Zur Hamatologie der beruflichen Strahlenschadigung I Follikel und Sinuslymphocyten. Dtsch. Gesundh. Wes *21* 826 (1966)

Adresse des Autors Prof Dr med ERHARD AMBS Universitats Kinderklinik, Josef Schneider Str 2, *8700 Würzburg* (BRD)

Acta Haemat 45 116-124 (1972)

Platelet Factor 3 in Glanzmann's Thrombasthenia

A K Saraya, Jaya Kasturi and Ram Kishan

Department of Pathology, All India Institute of Medical Sciences New Delhi

Abstract The present paper describes 3 cases of classical thrombasthenia and 3 cases of combined platelet factor 3 deficiency and absence of platelet aggregation The bleeding time and the clot retraction in the latter 3 patients were normal They, however did not differ in clinical presentation of classical thrombasthenia. These cases have been designated as thrombopathic thrombasthenia The possibility of an *in vivo* platelet activation leading to variable amounts of platelet factor 3 release in thrombasthenia has been raised Mechanism of PF3 release *in vivo* in thrombasthenia however is not known and needs further study

Key Words
Platelet factor 3
Thrombasthenia
Thrombopathy

Glanzmann's thrombasthenia (GT) is a clinical syndrome characterised by bleeding tendency with a prolonged bleeding time, defective clot retraction but normal platelet count [1, 2] Adenosine diphosphate (ADP) or thrombin-induced platelet aggregation is absent in these patients [2–4] The disease has been classified into mild, moderately severe and severe types according to intensity of bleeding and presence of one or more of the above mentioned abnormal findings [4–6] More recently, Papayannis and Israels [7] and Karaca and Nilsson [8] have concluded that clot retraction is an important finding and may be the only means of diagnosing a heterozygous form of thrombasthenia Platelet factor 3 (PF3) release is abnormal in GT [2, 3, 9–12] However, these reports do not comment on its total content in platelets Failure of platelet aggregation has been considered the cause of poor PF3 release [13] However, the detailed mechanism of its release in thrombasthenia needs to be studied further [14]

The present communication describes 6 patients with bleeding diathesis with normal platelet count and absence of platelet aggregation

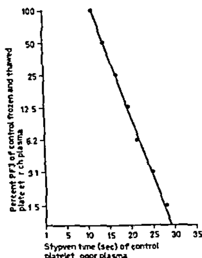


Fig 1 Stypven time (sec) of normal platelet poor plasma in relation to PF3 concentration (%) of frozen and thawed platelet rich plasma

with variable bleeding time and clot retraction Poor PF3 release on ADP stimulation has been noted in 3 and in others maximal PF3 release had occurred without ADP stimulation Reduced total PF3 contents of platelets were seen in 3 patients In those, bleeding time and clot retraction were normal It has been suggested that these cases are within the spectrum of GT and have been designated as thrombopathic thrombasthenia

Material and Methods

The patients have been attending the Haematology Clinic at All India Institute of Medical Sciences They primarily sought advice for abnormal bleeding A detailed clinical history and careful clinical examination was carried out. Care was taken to go into the details of family history with regard to bleeding diathesis Screening tests for haemostasis were done in each of these 6 patients A detailed study of platelet functions was then carried out.

Screening coagulation tests [15] were done in all patients Platelet count was done using formol citrate red cell diluent [16] Bleeding time was performed by the MIELKE *et al.* modification of the IVY method [17] Whole blood coagulation

time [18] one stage plasma prothrombin time [19] serum prothrombin time prothrombin consumption index (PCI) [20] clot retraction [21] partial thromboplastin time (PTT) and activated partial thromboplastin time [22] were done in each patient Stypven calcium time of platelet poor plasma (PPP) and platelet rich plasma (PRP) was done with 10 μg/ml of Russel viper venom Platelet aggregation was determined by a rapid qualitative test [16] after addition of ADP (10 μg/ml) adrenaline (1 25 μg/ml) noradrenaline (5 1 μg/ml) and thrombin (0.5 U/ml) Plateled factor 3 release was determined by the method of HARDISTY and HUTTON [23] Stypven time was noted with an aliquot of PRP with platelet count of 200 000 mm³ and ADP 10 μg/ml at 0 time and at intervals of 5 min up to 20 min The PRP, ADP mixture was incubated at 37 °C in a water bath Defective release of PF3 was differentiated from its absolute deficiency by assaying the total PF3 contents of repeatedly frozen and thawed platelet rich plasma (FTPRP) and expressed as percent of control (Fig 1) [24] Platelet adhesiveness *in vivo* was measured [25]

Results

Clinical studies The clinical features are summarised in table I Six patients, 1 male and 5 females, between 13 and 58 years of age had symptoms of generalised bleeding of varying intensity Mucosal and subcutaneous bleeding have been recorded in all of them Epistaxis, bleeding from gums and *per vaginam* were seen in all 5 females Melaena and bleeding *per rectum* were present in the male patient Intensity of bleeding was very severe in 1 and severe in 3 cases These patients have received blood transfusions at several occasions The other 2 patients had mild and moderate bleeding respectively History of bleeding was recorded since birth or early childhood in 5 patients However, in one (S N) it appeared for the first time at menarche and manifested as prolonged menorrhagia Definite positive family history was present in only 2 (M H and R P) patients Studies on their family members, however, could not be done

Haematological studies Severe degree of anaemia (haemoglobin 3 7 g%) has been recorded in 1 patient (S A) It was of moderate degree in 1 (R P) and mild in 2 (T C and M S) patients, respectively Screening tests of haemostasis are summarised in table II Platelet count and morphology were normal in 5 patients and were seen lying singly, clumps or small aggregates were not seen In 1 case (M S) mild thrombocytopoenia and large bizarre platelets with well demarcated granulomere and hyalomere were seen Markedly abnormal bleeding time (>15 min) and poor clot retraction were seen in 3 patients (S A , T C

Table I Clinical symptoms and family history of 6 patients with thrombasthenia

	S. A 13F	R. P 35M	T. C. 58F	M. S 15F	M. H 58F	S. N 35F
Bleeding						
Site	nose gums menorrhagia *bruising*	nose gums *per rectum* *bruising* melaena unusual bleeding from cuts	nose gums menorrhagia	nose gums menorrhagia *bruising*	nose gums menorrhagia *bruising* haematuria after tooth extraction	menorrhagia *bruising*
Intensity	very severe	severe	severe	severe	moderate	mild
Duration	since birth	since childhood	since childhood	since childhood	since childhood	since menarche
Family history	nil	history of bleeding in one son	nil	nil	history of bleeding in mother	nil

and R. P) The latter was slightly abnormal in 1 (M H) and normal in 2 patients (M S and S N) Whole blood coagulation time, plasma prothrombin time, PTT and activated PTT were normal in 5 of these patients However, in 1 (T C) the activated PTT was higher than normal and was corrected with fresh and stored normal serum. Stypven calcium time (Russel viper venom, 10 μg/ml) of PRP and PPP was normal in 1 (M S), higher in 2 (S A and T C.) and low in 3 (R. P, M H and S N) patients

Platelet adhesion was abnormal in 3 patients (T C., S A and S N) It was, however, considered normal (>20%) in the other 3 patients (table III) Platelet aggregation was uniformly absent with all reagents employed (ADP, adrenaline, noradrenaline, thrombin) in all the patients ADP induced platelet aggregation did not occur even with higher concentration of ADP (600 μg/ml)

PF3 was poorly released in 3 cases (T C., M S and S N) as compared to controls (fig 2) In 3 others (S N, M H and R. P), 0 min stypven time was well below that of controls and no PF3 release occurred with the addition of ADP Total PF3 contents on assay were 47,

Table II Screening tests for haemostasis in 6 patients of thrombasthenia

	S A	R P	T C	M S	M H	S N
Platelet count mm³	160 000	150 000	170 000	114 000	300 000	180 000
Bleeding time min	15	15	15	6	4	4
Clotting time min	9	6	10	9	10 5	9
Plasma prothrombin time, sec[1]	12/12	12/12.5	12/11	13/13	12/13	11/11
Partial thromboplastin time sec[1]	72/76	63/78	78/72	78/80	73/72	88/88
Activated partial thromboplastin time, sec[1]	37/41	33/43	49 42	38/40	36/40	32/33
Prothrombin consumption index %	44 4	53 8	66 6	86 6	40	79
Clot retraction %	20	14	22	50	38	48
Russel viper venom time sec[1] PPP[2] (mean 30 2, SD ± 3 5)	35/32	16/27	43/28	29/31	21/28	24/31
PRP (mean 24 8 SD ± 2.22)	30/26	15/24	36/24	25/21	18/23	20/25

[1] Patient normal

[2] Mean and 1 standard deviation Russel viper venom calcium time in 90 controls

Table III Platelet adhesion and PF3 assay in patients of thrombasthenia

	S A	R P	T C	M S	M H	S N
In vivo platelet adhesion %	0	20	11 7	28	25 6	16 6
PF3 assay, % of control	114	100	100	47	22	42

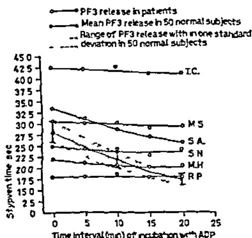


Fig 2 Platelet factor 3 release at 0, 5 10, 15 and 20 min after incubation with ADP in 6 patients of thrombasthenia and range with 1 standard deviation of PF3 release in 50 normal subjects.

22 and 42% of normal in M S, M H and S N, respectively It was 114% of normal in a single patient (S A), in the other 2 patients (T. C and S N) it was 100% (table III)

Discussion

These 6 patients with a history of bleeding of long duration without any systemic disease showed absence of platelet aggregation with ADP, adrenaline, noradrenaline and thrombin Classical features of GT such as normal platelet count and activated PTT, increased bleeding time, poor clot retraction and PF3 release, however, were seen in 3 patients PF3 release maximally had occurred without ADP stimulation in 1 case and was within normal range Total contents of PF3 in platelets were not reduced in these patients

Absence of platelet aggregation with normal bleeding time and clot retraction was seen in the other 3 patients. One of these also had mild thrombocytopenia with giant platelets in blood smear PF3 was poorly released in 1 case on ADP stimulation It had maximally been released in the other 2 patients prior to ADP stimulation However, it was less

than the PF3 released in normals Total PF3 contents of platelets also were significantly reduced in these 3 patients It can be argued that reduction of total PF3 contents of platelets in these cases is due to its excessive release in plasma However, the stypven time of PPP in R P, though being shortest, was not accompanied with PF3 deficiency Further, PF3 deficiency was seen in M S with normal stypven time of PPP indicating that PF3 has not been excessively released in plasma This compound defect of platelet PF3 deficiency with absence of platelet aggregation with clinical presentation of classical thrombasthenia has been designated as thrombopathic thrombasthenia Usage of this term had earlier been done in GT associated with poor PF3 release but with no reduction in its total amount in platelets [26] However, poor PF3 release in GT noted by various workers [2, 3, 9–12] has been recognised as a constituent defect of the disorder [27] Congenital [27–29] and hereditary [30] PF3 deficiency (deficit thrombopathy) associated with severe bleeding have been described and are considered as rare platelet disorders Congenital PF3 deficiency associated with absence of aggregation presently seen has not yet been reported However, a similar acquired platelet disorder (acquired thrombasthenic deficit thrombopathy) associated with macroglobulinaemia has recently been described [24]

Mechanism of PF3 release *in vitro* and *in vivo* is not fully understood Failure of platelet aggregation have been considered as the possible cause of poor PF3 release [13] However, poor PF3 release with normal aggregation have been noted in congenital [29] and acquired functional thrombopathies [31] Studies of HOROWITZ and PAPAYOANOU [14] also suggest that platelet agcregation and PF3 release are two independent reactions Poor PF3 release in 2 patients in whom total PF3 contents were normal, however, can be attributed to absence of platelet aggregation Mechanism of PF3 release, in 3 patients where it occurred prior to ADP stimulation as indicated by shorter stypven time of PPP (table II), is not clear An *in vivo* stimulation by activated coagulation factors or some unknown mechanism resulting in PF3 release from platelets are two possibilities Both these mechanisms, however, need further elucidation In presence of normal PTT and activated PTT the possibility of some activated coagulation factor responsible for PF3 release appears unlikely Stimuli such as thrombin, collagen and antigen antibody complexes, are known to release PF3 normally *in vivo* [32] Whether thrombin or collagen stimulation can lead to PF3 release in some cases of thrombasthenia, however, remains to be studied

It is concluded that thrombasthenia now covers a wide range of platelet disorders characterised by absence of platelet aggregation to various aggregating agents generally employed Rarely an associated deficiency of PF3 may occur with or without poor clot retraction and increased bleeding time This, however, would be considered a compound platelet defect designated here as thrombopathic thrombasthenia within the enlarged spectrum of Glanzmann's thrombasthenia

References

1 GLANZMANN, E. Hereditare hamorrhagische Thrombasthenie Ein Beitrag zur Pathologie der Blutplattchen. Jb Kinderheilk. *88* 1 (1918)

2 HARDISTY R. M, DORMANDY K. M., and HUTTON, R. U.. Thrombasthenia. Studies on three cases. Brit. J Haemat. *10* 371 (1964)

3 CAEN, J P CASTALDI P A. LECLERC, J C. INCEMAN, S LARRIEU M J, PROBST M., and BERNARD J Congenital bleeding disorders with long bleeding time and normal platelet count. I Glanzmann s thrombasthenia (report of fifteen patients) Amer J Med *41* 4 (1966)

4 CRONBERG S NILSSON I M., and ZETTERQVIST, E. Investigation of a family with members with both severe and mild degree of thrombasthenia. Acta paediat. scand *56* 189 (1967)

5 CRONBERG S and NILSSON, I. M.. Investigations in a family with thrombasthenia of moderately severe type with 16 affected members. Scand. J Haemat. *5* 17 (1968)

6 CRONBERG, S and NILSSON I M Investigation of patients with mild thrombasthenia A haemorrhagic disorder with prolonged bleeding time probably due to a primary platelet defect Acta med scand. *183* 163 (1968)

7 PAPAYANNIS A G and ISRAELS, M G G Glanzmann s disease and trait. Lancet *ii* 44 (1970)

8 KARACA, M and NILSSON I M Diagnosis of mild thrombasthenia. Lancet *ii* 1084 (1970)

9 CASTALDI, P A. LARRIEU M J., and CAEN, J Availability of platelet factor 3 and activation of factor XII in thrombasthenia. Nature, Lond. *207* 422 (1965)

10 WEISS H J and KOCHWA, S Studies of platelet function and proteins in 3 patients with Glanzmann s thrombasthenia J Lab clin. Med *71* 153 (1968)

11 INCEMAN S and TANGUN Y Platelet factor 3 availability in a patient with Glanzmann s disease Lancet *ii* 412 (1968)

12 INCEMAN, S and TANGUN Y Platelet factor 3 availability in Glanzmann s disease. Israel J med. Sci *5* *1259 (1969)*

13 MARCUS A. J Platelet function New Engl J Med. *280* 1213 1278, 1330 (1969)

14 HOROWITZ, H. I and PAPAYOANOU, M F Activation of platelet factor 3 by adenosine 5 diphosphate Thromb Diath haemorrh. *19* 18 (1968)

15 BIGGS, R. The detection of defects in blood coagulation Brit J Haemat. *15* 115 (1968)
16 DACIE, J V and LEWIS, S M Practical haematology, pp 70 321–323 (Churchill, London 1968)
17 MIELKE, C. H, KANESHIRO, M M, MATHER, I A, WEINER, J M, and RAPPAPORT, S I The standardized Ivy bleeding time and its prolongation by aspirin Blood *34* 204 (1969)
18 LEE, R I and WHITE, P D A clinical study of the coagulation time of blood Amer J med Sci *145* 495 (1913)
19 QUICK, A J The haemorrhagic diseases and the physiology of haemostasis (Thomas, Springfield 1942)
20 CARTWRIGHT, G E. Diagnostic laboratory haematology, pp 164–166 (Grune & Stratton, New York 1963)
21 MACFARLANE, R G A simple method for measuring clot retraction Lancet *i* 1199 (1939)
22 NYE, S W, GRAHAM, J B, and BRINKHOUS, K. M The partial thromboplastin time as a screening test for the detection of latent bleeding Amer J med Sci *243* 279 (1962)
23 HARDISTY, R M and HUTTON, R Platelet aggregation and the availability of platelet factor 3 Brit J Haemat *12* 768 (1966)
24 SARAYA, A K, KASTURI, JAYA, and KISHAN, R A study of haemostasis in macroglobulinaemia Acta haemat., Basel *47* 33 (1972)
25 BORCHGREVINK, C F A method for measuring platelet adhesiveness *in vivo* Acta med scand *168* 157 (1960)
26 ULUTIN, O N Qualitative platelet diseases Turk J Pediat *5* 237 (1963) quoted by BOWIE and OWEN [27]
27 BOWIE, E J W and OWEN, C A., jr Thrombopathy Sem Haemat *5* 73 (1968)
28 WEISS, H J and EICHELBERGER, J W., jr The detection of platelet defects in patients with mild bleeding disorders Use of a quantitative assay for platelet factor 3 Amer J Med *32* 872 (1962)
29 BOWIE, E J W, THOMPSON J H., jr., and OWEN, C. A., jr The blood platelet (including a discussion of qualitative platelet diseases) Mayo Clin Proc *40* 625 (1965)
30 KASTURI, J, SARAYA, A K., and KISHAN, R A study of platelet function in primary thrombopathy Ind J med Res (accepted for publication, 1972)
31 PACHTER, M R, JOHNSON, S A., NEBLETT, T R., and TRUANT, J P Bleeding platelets and macroglobulinaemia Amer J clin Path *31* 467 (1959)
32 PACKHAM, M A and MUSTARD, J F Platelet reactions Sem Haemat *8* 32 (1971)

Authors address Dr A. K. SARAYA, Dr JAYA KASTURI and Mr RAM KISHAN, Department of Pathology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 16 (India)

Acta Haemat 48 125–128 (1972)

The Distribution Pattern of Radioactive Ferrioxamine Administered Intravenously in Rats

Part I

M M Bobeck-Rutsaert, W F Wiltink, A M Op den Kelder, H G van Eijk and B Leijnse

Departments of Internal Medicine I and Chemical Pathology Medical Faculty, Rotterdam

Abstract Radioactive ^{59}Fe ferrioxamine was intravenously administered in rats The distribution of the radioactive label over different organs was examined. The injected ^{59}Fe ferrioxamine was partly (50–80%) excreted in the urine with a lower specific activity The decrease of the specific activity of ferrioxamine in the urine can be explained by an exchange between ferrioxamine iron and tissue iron

Key Words
Ferrioxamine
Iron metabolism

Many experiments have been described [1, 2] about the source of iron chelated by desferrioxamine Its origin is exclusively non haem iron, but the sites of chelation are not yet determined About the distribution, excretion pattern and exchange of ferrioxamine exist different opinions It has been reported for rats that ferrioxamine injected is stable in the organism and is excreted unchanged in the urine for 90–95%, within the first day [3, 4] However, utilization of ferrioxamine iron for haemoglobin-synthesis has also been reported, which implies a retention of part of the injected ferrioxamine [5] The experiments of Fielding [6] resulted in urinary ferrioxamine excretion of 50–70% of the intravenous injected dose after 72 h The rest of the iron is retained, but without exchange between ferrioxamine iron and other body iron The purpose of our investigations was to follow the distribution and excretion pattern of ferrioxamine in rats, in order to know whether it is fully excreted, retained or exchanged with other body iron

Materials and Methods

Adult Wistar male rats weighing between 250 and 300 g were used Desferrioxamine methane sulphonate (Desferal, Ciba) was supplied in sterile vials containing 500 mg crystallized powder, which was dissolved in isotonic saline, immediately prior to use In order to obtain wholly saturated desferrioxamine 20 mg Desferal was incubated with ^{59}Fe, labelled with a tracer amount $^{59}FeCl_3$ The ferrioxamine was gel filtrated over a Sephadex G25 column in a borate buffer pH 8.5, in order to remove traces of free ionogen iron from the ferrioxamine

59 Fe was measured in a gamma-counting apparatus (Philips PW 4280 01-4237 4002) The urinary iron was determined according to the method of TAVENIER et al [7] The basic principle is that in a strong acid solution the Fe(III) is quantitatively extractable with methyl isobutyl ketone Adding water to the MIBK layer, the Fe(III) is transferred into the water phase After removal of the MIBK layer, the desired chemicals are added to form the Fe CNS complex, which can be measured The total non haem iron content of the tissues was determined chemically by the wet destruction methon The homogenized tissues were destructed with perchloric acid After reduction, the iron content was measured spectrophotometrically at 535 nm When desferrioxamine is present in urine, it will be converted, at an acid pH in the presences of Fe(III) ions into the ferrioxamine iron complex Ferrioxamine was extracted quantitatively into benzyl alcohol from aqueous solutions saturated with NaCl and estimated spectrophotometrically at 430 nm [8]

Results

In order to gain insight into the behaviour pattern of completely saturated DF we administered intravenously radioactive ferrioxamine Rats were kept in metabolic cages, the 24-hour urine was carefully collected in iron-free glassware The distribution pattern of the injected ferrioxamine was followed The results are given in table I The extreme values of 10 experiments are given in table II

As table II shows, 50–80% of the labelled ferrioxamine was excreted in the urine on the first day This value is in contradiction to the value of 90–98%, mentioned by WÖHLER [3] and KEBERLE [4] The specific activity of ferrioxamine in a 24-hour urine sample after administration was about 20% decreased with respect to the sample injected, expressed as counts/100 sec/μmol Evidently there is an exchange between the ferrioxamine iron and other body iron The fact that all ferrioxamine should appear unchanged in the urine (98%) cannot be proven by our investigations

Table I Example of distribution of radioactive ferrioxamine in the organs of the rat, expressed as percentages of the administered dose

	Injected dose based on activity, %
Urine	69
Erythrocytes	5
Liver	2
Spleen	0 07
Intestine	0 10
Brain	0 01
Muscle	12
Bone	5
Faeces	4

Table II Distribution pattern of ^{59}Fe-ferrioxamine in 10 rats The extreme values are given

	Injected dose based on activity, %
Urine	50–80
Erythrocytes	2–6
Liver	1–2
Spleen	0 05–0 1
Intestine	0 09–0 13
Brain	0 01
Muscle	9–13
Bone	3–5
Faeces	4

Discussion

The distribution pattern of radioactive ferrioxamine was repeatedly followed It has been reported [3] that all ferrioxamine given by intramuscular or intravenous injection appears in the urine However, utilization of ferrioxamine iron for haemoglobin synthesis has also been reported [5] These conflicting data prompted us to study the distribution pattern After an intravenous injection of ferrioxamine, 50–80% was excreted in the urine in the first 24 h These results agree with the finding

of FIELDING [6] There is an incorporation of iron from ferrioxamine in the tissue iron The decrease of 20% of the specific activity in the urine may be explained by an exchange between ferrioxamine iron and tissue iron It is important to know to what extent ferrioxamine can exchange its iron with body iron, since the chelator desferrioxamine is clinically used in treatment of iron-overloading diseases Studies on the chelating with desferrioxamine in normal, anaemic and iron-overloaded rats will be described in a second paper

Acknowledgement The technical assistance of Mr P BACKHUYS is gratefully acknowledged

References

1 BROWN E. B YUN FEI HWANG and ALLGOOD J W Studies on the site of action of desferrioxamine J Lab clin Med *69* 382 (1967)
2 CUMMING R. L. C. MILLAR, J A SMITH, J A., and GOLDBERG A.. Clinical and laboratory studies on the action of desferrioxamine Brit J Haemat *17* 257 (1969)
3 WÖHLER F The treatment of haemachromatosis with desferrioxamine Acta haemat., Basel *30* 65 (1963)
4 KEBERLE, H The biochemistry of DF and its relation to iron metabolism Ann N Y Acad Sci *119* 758 (1964)
5 BANNERMAN, R. M CALLENDER S T., and WILLIAMS D L. Effect of DF and DTPA in iron overload Brit. med. J 2 1573 (1963)
6 FIELDING J Differential ferrioxamine test for measuring chelatable body iron J clin Path *18* 88 (1965)
7 TAVENIER P, RAAIJMAKERS C. E EIJK, H G VAN and LEIJNSE, B Determination of iron and transferrin in normal human urines Clin chim Acta *32* 63 (1971)
8 PLOEM J E Diagnostische proef voor ijzerstapeling bij de mens (Van Gorcum Assen 1965)

Author's address Dr M M BOBECK RUTSAERT, Dr W F WILTINK, Miss A. M OP DEN KELDER Dr H G VAN EIJK and Dr B LEIJNSE, Department of Internal Medicine I and Chemical Pathology Medical Faculty Rotterdam *Rotterdam* (The Netherlands)

Acta Haemat 48 129 136 (1972)

Biosynthesis *in vitro* of Immunoglobulins in Primary Immunological Deficiencies

F AIUTI and L FONTANA

I Clinica Malattie Infettive and I Istituto Patologica Medica
Universita di Roma, Roma

Abstract The biosynthesis *in vitro* of immunoglobulins was studied in 13 patients with primary immunological deficiencies Bone marrow tonsils, lymphocytes intestinal mucosa of congenital agammaglobulinaemic and acquired hypogammaglobulinaemic patients showed a very marked reduction of H and L immunoglobulin chains In 3 patients with dysgammaglobulinaemia there was a selective deficiency of the H chain restricted to classes lacking in the serum whereas in 1 patient with absence of IgA serum the tissue culture of lymphocytes was positive for this immunoglobulin These results are discussed with respect to the pathogenesis of immunological defects

Key Words
Dysgammaglobulinaemia
Hypogammaglobulinaemia
Immunoglobulin synthesis

The primary immunological deficiencies of the thymic independent system occur in two forms the inherited agammaglobulinaemia (X linked recessive and sporadic autosomal recessive affecting both males and females), and the primitive acquired hypogammaglobulinaemia in adults [5, 9, 10] Different pathogenetic mechanisms are suggested by the variability of the clinical picture, by the humoral antibody deficiency, and by the histopathological aspects of lymph nodes In addition, the lymphocytes of these patients in long term cultures synthesized immunoglobulins, short-term cultures were negative This behaviour suggests that the pathogenesis of the deficiency should occur in cells other than circulating lymphocytes [7, 15]

In an attempt to gain insight into the pathogenesis of the primary immunological defect, we studied the biosynthesis *in vitro* of immunoglobulins of different tissues of patients with agammaglobulinaemia and dysgammaglobulinaemia

Materials and Methods

Three cases of congenital agammaglobulinaemia, 6 cases of so-called acquired hypogammaglobulinaemia, and 4 cases of dysgammaglobulinaemia (2 with IgA and 2 with IgM deficiencies) were studied The diagnosis was based on anamnesis, clinical picture, peripheral blood and bone marrow smears, and tonsilar and intestinal mucosae biopsies

Tests of humoral antibody functions were also performed immunoquantitation of immunoglobulins [13], specific responses to Salmonella 0 and H antigens and tetanus and diphtheria toxoids. Skin tests to DNCB, PPD were made for the delayed hypersensitivity function The methods employed for these tests have been reported in previous articles [2]

The *in vitro* synthesis of immunoglobulins was performed by the tissue culture method of HOCHWALD *et al* [12] Tissue or cell suspensions were incubated for 48 h in rolling tubes in Eagle s medium, containing 1 μCi/ml of ^{14}C l lysine and 1 μCi/ml of ^{14}C-isoleucine The culture fluids after dialysis and lyophilization, were analysed by radioimmunoelectrophoresis developed with anti total serum proteins, anti γ-, anti-α, anti μ, anti $\varkappa$- and anti λ chains

In vitro synthesis was demonstrated by the autoradiographical immunoelectrophoretic pattern of the culture fluid The intensity of the autoradiographic lines, which indicate the amount of synthesized protein, were classified as follows – negative, +– slightly positive, + positive and ++ very positive The controls and the details of this method have been described in previous articles [2, 17]

Results

Cellular immunity was normal in all patients except in case 1, in which the PPD and DNCB were normal, but the *in vitro* response of the peripheral blood lymphocytes to PHA was decreased All subjects with X-linked and acquired agammaglobulinaemia failed to respond to TAB and tetanus diphtheria toxoid The isoagglutinin in these patients was negative

In subjects with dysgammaglobulinaemia antibody response was normal Table I shows the immunoglobulin levels and the results of the *in vitro* biosynthesis of γ-globulins of different patients with agammaglobulinaemia and dysgammaglobulinaemia None of the patients with congenital agammaglobulinaemia showed immunoglobulin production in all tissue cultures (fig 1) A weak synthesis of IgG and IgM was detected by radioimmunoelectrophoresis of bone marrow and of rectal mucosa culture fluids with respect to normal controls

Only 1 case of peripheral blood lymphocytic culture fluid was IgG positive (+–) (fig 2), constantly absent were the autoradiographic im-

Table I

Cases	Serum immunoglobulin levels mg 100 ml			Tissues	Radioimmunoelectrophoresis				
	IgG	IgA	IgM		IgG	IgA	IgM	κ	λ
Congenital agammaglobulinaemia									
S L	22	0	0	bone marrow	–	–	–	–	–
				lymphocytes	–	–	–	–	–
S V	32	0	0	bone marrow	–	–	–	–	–
				lymphocytes	–	–	–	–	–
				intestinal mucosa	–	–	–	–	–
				tonsils	–	–	–	–	–
R R	60	0	0	bone marrow	–	–	–	–	–
Acquired hypogammaglobulinaemia									
P A	27	0	0	bone marrow	+ –	+ –	–	+ –	+ –
				lymphocytes	–	–	–	–	–
				tonsils	–	–	–	–	–
G M	320	32	12	bone marrow	+ –	–	–	–	–
				lymphocytes	+ –	–	–	–	–
F A	186	21	32	bone marrow	–	–	+	+ –	+ –
				lymphocytes	–	–	–	–	–
T M	200	18	26	bone marrow	–	–	+	–	–
P B	220	12	4	bone marrow	+	–	–	–	–
				lymphocytes	–	–	–	–	–
				tonsils	–	–	–	–	–
B V	210	0	0	bone marrow	+ –	–	–	–	–
				lymphocytes	–	–	–	–	–
				rectal mucosa	+	–	+ –	+ –	+ –
Dysgammaglobulinaemia									
M M	940	12	54	lymphocytes	+ –	–	–	–	–
				rectal mucosa	+ –	–	+ –	–	–
U S	1 100	0	110	lymphocytes	+	+	+ –	–	–
C M	1 020	180	0	bone marrow	+	+	+ –	+ –	+ –
D S	750	240	0	bone marrow	+ +	+ +	–	+ –	+ –
Normal controls									
10 cases				bone marrow	+ + +	+ +	+ +	+	+
12 cases				lymphocytes	±	±	±	±	±
8 cases				rectal mucosa	±	+	±	±	+
12 cases				tonsils	+ + +	+ +	+ +	+	+







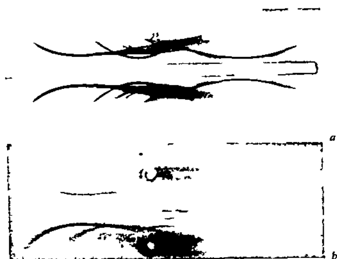


Fig 2 *a* Immunoelectrophoresis of carrier serum and normal bone marrow culture fluid (below) and peripheral blood lymphocyte culture fluid of a patient with acquired agammaglobulinaemia. *b* Autoradiography Very dark images of IgG IgA and IgM lines (below) and weak positive IgG line (above)

novo In the other case, IgM production was negative IgG and IgA were positive in both cases

Discussion

The results reveal that the immunocompetent cells of bone marrow, of peripheral blood of tonsils and of intestinal mucosa in patients with X-linked congenital and acquired agammaglobulinaemia show, as compared with normal controls a very marked reduction of immunoglobulin biosynthesis

These results are in agreement with the immunoglobulin levels in congenital agammaglobulinaemia In 4 cases with acquired agammaglobulinaemia there is a very small production of γ-globulins in agreement with immunoglobulin levels Nevertheless, in the same subjects instead of low immunoglobulin levels no synthesis was detected by autoradiographic studies This discrepancy could be explained by a very small incorporation of radioactive amino acids which could prevent its detection by radioimmunoelectrophoresis It should also be mentioned that in patients

with agammaglobulinaemia there is a remarkable prolongation of the half life of γ-globulin which could increase the serum levels [18]. The lack of IgA and IgG production by intestinal mucosa explain the defect of local immunity in these patients. The absence of IgA in intestinal culture fluid of patient B V with acquired hypogammaglobulinaemia is in part repaired by a local production of IgG. These data are in agreement with those reported by VAN FURTH *et al.* [17], FUDENBERG and HIRSHHORN [9] and ELVES *et al.* [8] who revealed that lymphocytes of hypogammaglobulinaemic patients failed to produce γ-globulin in short time cultures. These investigators support the idea of a defect of the structural or regulatory genes of immunoglobulins.

On the contrary, other experiences with long term lymphocyte cultures derived from patients with hypogammaglobulinaemia constantly showed a synthesis of IgG and IgM [7, 15]. These researchers maintain that lymphocytes do not retain the biochemical abnormalities of the cell donors and that the pathogenesis would probably appear in cells other than circulating lymphocytes. Our results with short term cultures demonstrated that the deficiency involved tissues (bone marrow, tonsils, intestinal mucosa) other than circulating lymphocytes.

In addition, our studies suggested a correlation between the immunoglobulin level and *in vitro* biosynthesis. In 3 patients with dysgammaglobulinaemia there was a correspondence between the serum γ-globulin value and the *in vitro* synthesis. Therefore, it would appear that in these subjects the selective deficiency of immunoglobulin production should only be attributed to a synthesis defect of the heavy chain, whereas on the contrary, light chains are produced *in vitro* although in limited quantities thus confirming that the error in the synthesis does not occur in this part of the antibody molecule. As regards patient U S the interpretation of the results is more complex because of a selective deficiency of the serum IgA with normal *in vitro* formation of IgA.

There are two possible assumptions: either IgA is synthesized only by the lymphocytes of peripheral blood and thus does not reach a large enough concentration in the serum, or the synthesis of this γ-globulin fraction is normal but its half life is extremely low. This second assumption could be indirectly confirmed by the observation made in 2 cases of IgA or IgM dysgammaglobulinaemia where an accelerated catabolism was found due to the presence of auto-antibodies anti IgA or IgM [4, 16].

Our results show that in X linked agammaglobulinaemia and in acquired hypogammaglobulinaemia the immunoglobulin defect is due to a

reduced or missed synthesis by the immunocompetent cells of different tissues It should be clarified whether or not this predominant defect is due to the absence of B lymphocytes, or to the incapacity of B lymphocytes to differentiate in plasma cells, or to a genetic defect operative *in vivo* and in short tissue cultures Our data does not permit to clarify the situation

New experiments performed on these patients [2] and in numerous agammaglobulinaemia by other researchers [6, 11, 14] seem to suggest at least two different conditions In patients with congenital agammaglobulinaemia or with severe acquired hypogammaglobulinaemia, there is a lack of B cells with surface immunoglobulins probably due to the absence of the stem cell or to a defect in the maturation of the stem cell Nevertheless, in patients with dysgammaglobulinaemia or mild hypogammaglobulinaemia with normal B cells in peripheral blood, the defect is probably due to the conversion of a B precursor cell in an antibody producing cell

Acknowledgments The authors wish to thank Dr P Guglielmetti for valuable comments on the English manuscript and the student M Fiorilli for excellent technical assistance This article is supported by a grant of Consiglio Nazionale delle Ricerche' Italy

References

1 Aiuti F Fontana L., and Gatti R B-cell function in primary immunodeficiencies Scand J Immunol (in press)
2 Aiuti F Turbessi G and Ugolini A Synthesis *in vitro* of immunoglobulins produced by different human mucous membranes Experientia *25* 1089–1091 (1969)
3 Aiuti F Furth R van Turbessi G e Ricci G Sintesi *in vitro* delle immunoglobuline nei tessuti degli apparati gastroenterico e respiratorio nella mucosa congiuntivale vaginale e nella cute Progr immunobiol Standard, vol 4 pp 297 303 (Karger Basel 1970)
4 Andersen S B Metabolism of gammaglobulin (gamma ss globulin) (Blackwell Oxford 1964)
5 Bruton O C Agammaglobulinemia Pediatrics 9 722–729 (1952)
6 Cooper M D Lawton A R., and Bockman D E Agammaglobulinaemia with B lymphocytes Lancet *ii* 791–794 (1971)
7 Cooperband S R Rosen F S., and Kibrick, S Studies on the *in vitro* behaviour of agammaglobulinemic lymphocytes J clin Invest *47* 836–847 (1968)
8 Elves M W Roath S and Israëls Mc G Failure of lymphocytes from hypogammaglobulinaemic subjects to transform in culture Brit. med J *ii* 1052–1054 (1964)

9 FUDENBERG H H and HIRSHHORN K Agammaglobulinemia the fundamental defect Science *145* 611–613 (1964)
10 GOOD R A and BERGSMAN J Immunologic deficiency diseases in man In Birth defects, vol 4 New York nat Found (1968)
11 GREY H M RABELLINO E, and PIROFSKY B Immunoglobulins on the surface of lymphocytes IV J clin Invest *50* 2368 2375 (1971)
12 HOCHWALD G M THORBECKE G J, and ASOFSKY R Sites of formation of immune globulins and of a component of C'3 J exp Med *114* 459–470 (1961)
13 MANCINI G CARBONARA A O, and HEREMANS J F Immunochemical quantitation of antigens by single radial immunodiffusion Immunochemistry *2* 235 243 (1965)
14 PAPAMICHAIL M BROWN J C, and HOLBOROW E J Immunoglobulins on the surface of human lymphocytes Lancet *ii* 850 853 (1971)
15 STITES D P, LEVIN A S AUSTIN K E, and FUDENBERG H Immunobiology of human lymphoid cell lines I J Immunol *107* 1376–1381 (1971)
16 STROBER W WOCHNER R D BARLOW M H MCFARLIN D E and WALDMANN T A Immunoglobulin metabolism in ataxia telangectasia J clin Invest *47* 1905–1915 (1968)
17 FURTH R VAN SCHUIT R E and HIJMANS W The formation of immunoglobulins by human tissue *in vitro* Spleen lymph nodes bone marrow and thymus Immunology Lond *11* 19 31 (1966)
18 WALDMANN T A and STROBER W Metabolism of immunoglobulins Progr Allergy vol 13 pp 1–110 (Karger Basel 1969)

Authors address Prof F Aiuti and Dr I Fontana I Clinica Malattie Infettive Policlinico Umberto I *I-00161 Roma* (Italy)

Acta Haemat 48 137-144 (1972)

Plasma Radioactivity Following Simultaneous Oral Administration of Intrinsic Factor-Bound and Free Radioactive Vitamin B_{12}

R D Finney and R W Payne

The Departments of Haematology, Glasgow Royal Infirmary, and the Victoria Infirmary, Glasgow

Abstract Plasma radioactivity following the simultaneous oral administration of vitamin B_{12} labelled with 2 isotopes of cobalt (Dicopac test) was measured in 19 control subjects and in 19 patients suffering from pernicious anaemia One isotope (^{58}Co) was free while the other (^{57}Co) was bound to normal human gastric juice Results of diagnostic value were obtained on 80% of occasions misleading results occurred in the other 20% It is concluded that this test is of little value in routine diagnostic haematology

Key Words
Dicopac' test
Intrinsic factor
Pernicious anaemia
Vitamin B_{12} absorption

The use of radioactive vitamin B_{12} absorption tests based on measurements of plasma radioactivity is well documented in normality, and in malabsorption of vitamin B_{12} [4, 6] It has been shown that there is excellent correlation between 8 hour peak levels of radioactivity in plasma and 24-hour urinary excretion, in both normal subjects and patients suffering from pernicious anaemia [5, 7] Determination of plasma radioactivity has several advantages over measurement of urinary excretion alone [8, 10]

A dual isotope (Dicopac) test for the investigation of suspected vitamin B_{12} malabsorption has recently become commercially available The basis of this test is the simultaneous administration of 2 isotopic forms of radioactive Co labelled vitamin B_{12} one of which is free and the other bound to normal human gastric juice Thus this test allows both the absorption of vitamin B_{12} and the effect of intrinsic factor to be assessed in a single diagnostic procedure

This study was undertaken to evaluate the 'Dicopac' test using measurement of levels of plasma radioactivity in control subjects and in pa-

tients with pernicious anaemia, we have already reported the results of an evaluation of this test using measurement of urinary excretion [11].

Materials and Methods

The dual radio-isotope preparation of vitamin B_{12} (Dicopac®) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire. There are 2 different capsules in the Dicopac test: one containing 0.25 μg vitamin B_{12} labelled with 0.8 μCi ^{58}Co and the other containing 0.25 μg vitamin B_{12} labelled with 0.5 μCi ^{57}Co and bound to normal human gastric juice. The kit also contains diluted standards of ^{58}Co and ^{57}Co and also a vial of 1,000 μg non-radioactive vitamin B_{12} (Cyanocobalamin BP).

After an overnight fast, 2 capsules containing free ^{58}Co-labelled vitamin B_{12} and 2 capsules ^{57}Co-labelled vitamin B_{12} bound to normal human gastric juice were given by mouth with about 100 ml of tap water. An intramuscular injection of a flushing dose of 1,000 μg of non-radioactive vitamin B_{12} was given 2 h later at 8 a.m. A 10-ml sample of venous blood was obtained at 4 p.m. and placed in a lithium heparin bottle (Stayne Laboratories Ltd.). After centrifugation, a 4 ml aliquot of plasma was retained for subsequent counting of radioactivity.

All measurements of radioactivity were made with a well-type scintillation counter (Ekco General Purpose Scintillation Detector M 5401) using a thallium activated sodium iodide crystal (Ekco well type 2 in diameter crystal). Counting rates at optimal settings for ^{58}Co and for ^{57}Co were determined for background radioactivity, for 4 ml aliquots of the 2 standards after dilution, and for the 4 ml aliquot of plasma.

Two groups, each containing 19 individuals, were studied.

Control subjects. The control group was made up of 19 hospital in-patients matched for age and sex with the group of patients suffering from pernicious anaemia. The control subjects showed no evidence of macrocytic anaemia, gastro-intestinal, hepatic or renal disease. These individuals had understood the nature of the test and agreed to its performance.

Pernicious anaemia. In this group of 19 hospital in-patients there was evidence of pernicious anaemia. All patients had a macrocytic anaemia and megaloblastic changes in the bone marrow with reduced serum vitamin B_{12} and normal serum 'folate' levels; histamine-fast achlorhydria was present. Subsequently a complete haematological response followed vitamin B_{12} therapy. There was no history of gastro-intestinal surgery nor was there any evidence of gastro-intestinal or renal disease. The mean age of the group was 67.3 years (range 36–82 years); 14 were female and 5 were males.

Results

In each group results have been expressed as follows: (1) the percentage of administered free ^{58}Co/l of plasma; (2) the percentage of ad-

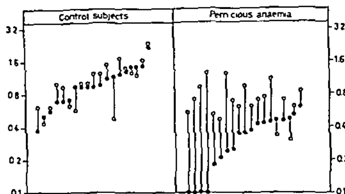


Fig 1 Plasma radioactivity (% administered dose/l of plasma) ● = ^{58}Co-vitamin B_{12} (free) ○ = ^{57}Co-vitamin B_{12} (bound)

Table I Summary of results of levels of plasma radioactivity in control subjects and in pernicious anaemia

		Control subjects	Pernicious anaemia
Free ^{58}Co	range	0 38–2.30	0 10–0 63
%/l	mean	1 08	0 34
	standard deviation	±0 45	±0 17
Bound ^{57}Co	range	0 44–2.41	0 30–1 34
%/l	mean	1 13	0 77
	standard deviation	±0.52	±0.28
Bound free	range	0 4–1 6	0 6–13 4
ratio	mean	1 1	3 5

ministered bound' ^{57}Co/l of plasma, (3) the ratio of these two percentages

These results are summarised in table I and shown in diagrammatic form in figure 1 and 2

Control subjects The mean percentage of free ^{58}Co was 1 08% administered dose/l of plasma (range 0 38–2 30%) The mean percentage of bound ^{57}Co was 1 13% administered dose/l of plasma (range 0 44–2 41%) The mean bound free ratio was 1 1 (range 0 4–1 6)

tients with pernicious anaemia, we have already reported the results of an evaluation of this test using measurement of urinary excretion [11]

Materials and Methods

The dual radio-isotope preparation of vitamin B_{12} (Dicopac) was obtained from the Radiochemical Centre Amersham Buckinghamshire There are 2 different capsules in the 'Dicopac' test, one containing 0.25 µg vitamin B_{12} labelled with 0.8 µCi ^{58}Co and the other containing 0.25 µg vitamin B_{12} labelled with 0.5 µCi ^{57}Co and bound to normal human gastric juice The kit also contains diluted standards of ^{57}Co and ^{58}Co and also a vial of 1000 µg non radioactive vitamin B_{12} (Cyanocobalamin BP)

After an overnight fast 2 capsules containing free ^{58}Co-labelled vitamin B_{12} and 2 capsules ^{57}Co-labelled vitamin B_{12} bound to normal human gastric juice were given by mouth with about 100 ml of tap water An intramuscular injection of a 'flushing dose' of 1000 µg of non radioactive vitamin B_{12} was given 2 h later at 8 a.m. A 10-ml sample of venous blood was obtained at 4 p.m. and placed in a lithium heparin bottle (Stayne Laboratories Ltd) After centrifugation a 4 ml aliquot of plasma was retained for subsequent counting of radioactivity

All measurements of radioactivity were made with a well type scintillation counter (Ecko General Purpose Scintillation Detector M 5401) using a thallium activated sodium iodide crystal (Ecko well type 2 in diameter crystal) Counting rates at optimal settings for ^{58}Co and for ^{57}Co were determined for background radioactivity for 4 ml aliquots of the 2 standards after dilution and for the 4 ml aliquot of plasma

Two groups, each containing 19 individuals were studied

Control subjects The control group was made up of 19 hospital in-patients matched for age and sex with the group of patients suffering from pernicious anaemia The control subjects showed no evidence of macrocytic anaemia gastro intestinal hepatic or renal disease These individuals had understood the nature of the test and agreed to its performance

Pernicious anaemia In this group of 19 hospital in-patients there was evidence of pernicious anaemia All patients had a macrocytic anaemia and megaloblastic changes in the bone marrow with reduced serum vitamin B_{12} and normal serum 'folate' levels histamine fast achlorhydria was present Subsequently a complete haematological response followed vitamin B_{12} therapy There was no history of gastro-intestinal surgery nor was there any evidence of gastro-intestinal or renal disease The mean age of the group was 62.3 years (range 36–82 years) 14 were female and 5 were males.

Results

In each group results have been expressed as follows (1) the percentage of administered free ^{58}Co % of plasma, (2) the percentage of ad-

Table II Subdivision of plasma radioactivity ranges in normal subjects and in pernicious anaemia

	^{58}Co plasma radioactivity % administered dose/l		
	below 0 45 %	0 45–0 60 %	above 0 60 %
Normal subjects	1	1	17
Pernicious anaemia	13	5	1
Total	14	6	18

Table III Analysis of diagnostic value of plasma radioactivity

Diagnostically correct	normal subjects	17	– 79 0 %
	pernicious anaemia	13	–
Equivocal results	normal subjects	1	– 15 8 %
	pernicious anaemia	5	–
False abnormal	normal subjects	1	2 6 %
False normal	pernicious anaemia	1	2 6 %

Table IV Subdivision of plasma bound free ratios in normal subjects and in pernicious anaemia

	Plasma $\frac{^{57}\text{Co}\ \%}{^{58}\text{Co}\ \%}$ ratio	
	0–1 6	1 7
Normal subjects	19	0
Pernicious anaemia	7	12
Total	26	12

equivocal results obtained on 16% of occasions (1 out of 19 normal subjects and 5 out of 19 patients with pernicious anaemia) (table III)

The bound free ratio of plasma radioactivity did not exceed 1 6 in any normal subject, and this has, therefore, been accepted as the upper

80% of occasions, on the other 20% diagnostically misleading information would have been obtained. This applied to both the free ^{58}Co vitamin B_{12} plasma levels, and also to the bound : free ratios.

These results, although disappointing, are not unduly surprising since recent studies have demonstrated the practical advantages of using vitamin B_{12} labelled with ^{57}Co rather than with ^{58}Co [8, 10]. This situation might well be improved by the use of free ^{57}Co-labelled vitamin B_{12} and ^{58}Co-labelled vitamin B_{12} bound to human gastric juice, (a reversal of the materials used in this study). Such a reversal of isotopes would subject the individual to a considerably increased dose of radiation, particularly to the liver, and is therefore undesirable.

For the above reasons we would not advocate the use of plasma radioactivity in the 'Dicopac' test. We prefer to measure plasma radioactivity using ^{57}Co labelled vitamin B_{12} with repetition of the test with added intrinsic factor where necessary [7].

Acknowledgement. We wish to thank Dr. R. J. BAYLY of the Radiochemical Centre for supplying the Dicopac tests used in this study. We also wish to thank Miss M. DAVIDSON for her technical assistance and interest. We are grateful to members of the medical and nursing staffs of the Victoria Infirmary and Royal Infirmary, Glasgow for their helpful co-operation.

References

1 ARMSTRONG, B. K. and WOODLIFF, H. J.: Studies on the ^{57}Co vitamin B_{12} plasma level absorption test. J. clin. Path. *23*: 569–571 (1970).
2 BELL, T. K.; BRIDGES, J. M., and NELSON, M. G.: Simultaneous free and bound radioactive vitamin B_{12} urinary excretion test. J. clin. Path. *18*: 611–613 (1965).
3 BELL, T. K. and LEE, D.: Evaluation of a dual radioisotope urinary excretion test in the diagnosis of pernicious anaemia. Acta haemat. Basel *42*: 183–187 (1969).
4 BOOTH, C. C. and MOLLIN, D. L.: Plasma, tissue and urinary radioactivity after oral administration of ^{58}Co labelled vitamin B_{12}. Brit. J. Haemat. *2*: 223–236 (1956).
5 DONALDSON, D.; BLIGHT, B. J. N., and LASCELLES, P. T.: An assessment of serum ^{57}Co cyanocobalamin as an index of vitamin B_{12} absorption. J. clin. Path. *23*: 558–562 (1970).
6 DOSCHERHOLMEN, A. and HAGEN, P. S.: Radioactive vitamin B_{12} absorption studies: results of direct measurement of radioactivity in blood. Blood *12*: 336–346 (1957).

7 FINNEY, R D and PAYNE R W Plasma radioactivity following oral administration of ^{57}Co-labelled vitamin B_{12} Scot med J *13* 425–429 (1968)
8 FORSHAW, J and HARWOOD, L. Measurement of intestinal absorption of ^{57}Co vitamin B_{12} by serum counting J clin Path *19* 606–609 (1966)
9 KATZ, J H, DIMASE, J., and DONALDSON R M, jr Simultaneous administration of gastric juice bound and free radioactive cyanocobalamin rapid procedure for differentiating between intrinsic factor deficiency and other causes of vitamin B_{12} malabsorption J Lab clin Med *61* 266–271 (1963)
10 NEEF W B, MCAFEE J G., and WAGNER H N, jr Single measurement of plasma radioactive vitamin B_{12} as a test for pernicious anaemia J Lab clin Med *61* 158–165 (1963)
11 PAYNE R W and FINNEY R D An evaluation of the 'Dicopac' test in control subjects and in pernicious anaemia (to be published)

Authors addresses Dr R D FINNEY Haematology Department Royal Infirmary *Glasgow G4 0SF* and Dr R W PAYNE Haematology Department Victoria Infirmary *Glasgow G42 2TY* (UK)

Acta Haemat 48 145-150 (1972)

New Carrier Protein(s) of Folic Acid in Human Serum

T Markkanen, R -L Pajula, S Virtanen and P Himanen

Department of Medical Microbiology University of Turku, Turku

Abstract α_2 Macroglobulin a carrier of folic acid activity (FAA) was isolated from fresh human serum by DEAE Sephadex A 50 gel chromatography The FAA content of the maximum with α_2 macroglobulin was 136 pg/mg protein and constituted 42% of the FAA eluated with the total proteins from the chromatography column The results was corroborated by controlled retesting

Key Words
α_2 Macroglobulin
Carrier proteins of folic acid
Folic acid metabolism
Gel chromatography
Serum folic acid

It has previously been reported from our laboratory that the folic acid activity (FAA) of plain human serum is divided, in Sephadex G 25 chromatography, into so-called free and protein bound fractions, and that the latter in Sephadex G 200 chromatography is divided into 2 maxima The first bound fraction is bound to high molecular proteins (globulins), and the second FAA fraction to small molecular proteins (the size of albumin molecules) [2, 3, 6, 7] Later we are able to purify the last mentioned, FAA-carrying protein fraction, and on immunological analysis it proved to be albumin [5] We have also shown that the FAA follows these rules of binding in other organic fluids as well Most of the FAA in cerebrospinal fluid however, is not protein bound [6] Measurable amounts of FAA are fixed in the proteins secreted into the urine of patients with proteinuria [7]

Another FAA carrier protein, isolated in our continued studies from the globulin area of the protein spectrum of the serum, is described below

Material and Methods

Venous blood samples were taken for chromatography and treated as described earlier [5]. Special care was taken to apply serum samples to the chromatography column immediately after the sample had been taken and centrifuged, without any protective agents which we have found to split FAA from its protein bound [2]. All donors of the samples were young healthy men under 25 years of age.

In the procedure for the isolation of the new carrier protein, we adopted techniques different from those described earlier [5]. DEAE Sephadex A 50 gel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was used for chromatography, and a thin layer of Sephadex G 25 coarse gel (the same manufacturer) was placed at the bottom of the column. The last mentioned gel was put to swell for 3 h in distilled water and DEAE Sephadex A 50 for 3 days in the specific packaging buffer at room temperature, protected against impurities of the air. After the chromatographic process, the same gel was regenerated by elution with 2.5 M NaCl buffer solution and 0.1 N NaOH. Subsequently, the gel was rinsed with distilled water and retransferred for a fresh packing into packaging buffer. Column size 40 × 500 mm.

Buffers: the basic buffer was 0.05 M sodium phosphate buffer, pH 7.0. (Solution A: 0.5 M Na_2HPO_4, 71 g/l. Solution B: 0.5 M $NaH_2PO_4 \times H_2O$, 69 g/l. Solution A was added to solution B, and the pH was adjusted to 7.0. Dilution 1:10.) The packaging buffer was 0.05 M sodium phosphate buffer, pH 7.0, with 0.02 M NaCl solution. The elution buffer consisted of solutions obtained by adding NaCl to the basic buffer: 0.05, 0.10, 0.15, 0.20, 0.30, 0.40 and 0.50 M.

To eliminate bacterial contamination which is highly detrimental to FAA chromatography, all solutions were sterilized for 15 min at 120 °C in an autoclave and kept in stoppered flasks in the refrigerator at +4 °C. In this way, bacterial invasion into chromatography gel during the run could be totally prevented. All the chemicals represented the purity degree 'pro analysi' (Merck).

Serum chromatography: 40 ml serum was applied to the column. After serum absorption, 200 ml of the packaging buffer containing 0.02 M NaCl, was applied to the column.

The serum was eluated from the column by stages with buffer solutions with NaCl concentrations increasing from 0.02 to 0.50 M. The elution buffers, 250 ml of each, were added by pairs consecutively to the mixing flask (the true elution flask) and to the subsidiary flask. The flasks were connected with tubing.

Fractionation was carried out at +10 °C under ultraviolet light. The 10-ml fractions were collected with an automatic collector (LKB RadiRac). The rate of elution was 50 ml/h with fresh gel and 100 ml/h with regenerated gel. The fractions, in protected tubes, were immediately placed into a dark refrigerator and their treatment was started at once.

Proteins were determined by means of the biuret reaction, as described previously, and the FAA of the fractions was determined microbiologically with the *L. casei* method also described previously [5]. The immunologic identification of the carrier proteins was also carried out as described before [5]. The control preparations used were the standards of Behringwerke AG. The results are presented in figure 1 and table 1.

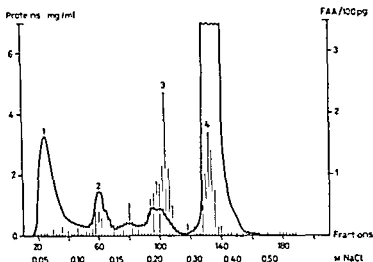


Fig. 1. DEAE Sephadex A 50 chromatography of the serum of a 25 year-old healthy man. Protein content 76.4 mg/ml. Total FAA 4.1 ng/ml, of which about 35% was eluated from the column together with proteins. Saline solutions 0.020–0.150 M NaCl were eluated separately by stages, from the column, and 0.200–0.500 M NaCl were eluated by pairs as described in chapter Material and Methods. The protein maxima are numbered 1–4 in the diagram. The contents of these maxima are described in table I.

Results

Figure 1 shows that with the described techniques of chromatography human serum was divided into 4 principal protein maxima: the first contained pure γ-globulin, the second transferrin and a γ-globulin fraction so far unidentified; in the third protein maximum the FAA peak showed pure α_2 macroglobulin and in the fourth albumin.

Table I shows how FAA is bound to each protein maximum per mg protein. It also gives the FAA contained in each protein maximum as a percentage of the FAA of the total protein area. The α_2 macroglobulin was found to be proportionally the greatest, viz. 42% of all bound FAA. The second place was held by albumin with 22% of the bound FAA. Third came the protein maximum which definitely contains transferrin and additionally a γ-globulin fraction so far unidentified. Studies concern-

Material and Methods

Venous blood samples were taken for chromatography and treated as described earlier [5]. Special care was taken to apply serum samples to the chromatography column immediately after the sample had been taken and centrifuged, without any protective agents which we have found to split FAA from its protein bound [2]. All donors of the samples were young healthy men under 25 years of age.

In the procedure for the isolation of the new carrier protein, we adopted techniques different from those described earlier [5]. DEAE Sephadex A 50 gel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was used for chromatography, and a thin layer of Sephadex G 25 coarse gel (the same manufacturer) was placed at the bottom of the column. The last mentioned gel was put to swell for 3 h in distilled water and DEAE Sephadex A 50 for 3 days in the specific packaging buffer, at room temperature, protected against impurities of the air. After the chromatgoraphic process, the same gel was regenerated by elution with 2.5 M NaCl buffer solution and 0.1 N NaOH. Subsequently, the gel was rinsed with distilled water and retransferred for a fresh packing into packaging buffer. Column size: 40 × 500 mm.

Buffers: the basic buffer was 0.05 M sodium phosphate buffer, pH 7.0 (Solution A: 0.5 M Na_2HPO_4, 71 g/l. Solution B: 0.5 M $NaH_2PO_4 \times H_2O$, 69 g/l. Solution A was added to solution B and the pH was adjusted to 7.0. Dilution 1:10.) The packaging buffer was 0.05 M sodium phosphate buffer, pH 7.0, with 0.02 M NaCl solution. The elution buffer consisted of solutions obtained by adding NaCl to the basic buffer: 0.05, 0.10, 0.15, 0.20, 0.30, 0.40 and 0.50 M.

To eliminate bacterial contamination, which is highly detrimental to FAA chromatography, all solutions were sterilized for 15 min at 120 °C in an autoclave and kept in stoppered flasks in the refrigerator at +4 °C. In this way, bacterial invasion into chromatography gel during the run could be totally prevented. All the chemicals represented the purity degree 'pro analysi' (Merck).

Serum chromatography: 40 ml serum was applied to the column. After serum absorption, 200 ml of the packaging buffer containing 0.02 M NaCl, was applied to the column.

The serum was eluated from the column by stages, with buffer solutions with NaCl concentrations increasing from 0.02 to 0.50 M. The elution buffers, 250 ml of each, were added by pairs, consecutively to the mixing flask (the true elution flask) and to the subsidiary flask. The flasks were connected with tubing.

Fractionation was carried out at +10 °C under ultraviolet light. The 10-ml fractions were collected with an automatic collector (LKB RadiRac). The rate of elution was 50 ml/h with fresh gel and 100 ml/h with regenerated gel. The fractions in protected tubes, were immediately placed into a dark refrigerator and their treatment was started at once.

Proteins were determined by means of the biuret reaction, as described previously, and the FAA of the fractions was determined microbiologically with the *L. casei* method also described previously [5]. The immunologic identification of the carrier proteins was also carried out as described before [5]. The control preparations used were the standards of Behringwerke AG. The results are presented in figure 1 and table I.

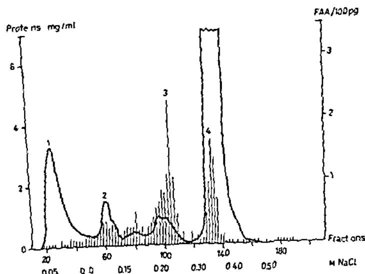


Fig 1 DEAE Sephadex A 50 chromatography of the serum of a 25 year-old healthy man Protein content 76 4 mg ml Total FAA 4 1 ng/ml of which about 35% was eluated from the column together with proteins Saline solutions 0 020–0 150 M NaCl were eluated separately by stages from the column and 0 200–0 500 M NaCl were eluated by parts as described in chapter Material and Methods The protein maxima are numbered 1–4 in the diagram The contents of these maxima are described in table I

Results

Figure 1 shows that with the described techniques of chromatography human serum was divided into 4 principal protein maxima the first contained pure γ-globulin the second transferrin and a γ-globulin fraction so far unidentified in the third protein maximum the FAA peak showed pure α_2 macroglobulin and in the fourth albumin

Table I shows how FAA is bound to each protein maximum per mg protein It also gives the FAA contained in each protein maximum as a percentage of the FAA of the total protein area The α_2 macroglobulin was found to be proportionally the greatest viz. 42% of all bound FAA The second place was held by albumin with 22% of the bound FAA Third came the protein maximum which definitely contains transferrin and additionally a γ-globulin fraction so far unidentified Studies concern

Table I Details of the protein components and FAA contents of the protein maxima 1–4 of figure 1 It should be noted that FAA occurs in the intervals between maxima, and the maxima show only 79% of the protein bound FAA eluated from the column α_2-macroglubolin binds the most of the serum FAA

Protein maxima in chromatography	Carrier protein(s)	pg FAA mg protein	FAA percent of recovery
1st	γ-globulin	4	3
2nd	transferrin + γ-globulin fraction	38	12
3th	α_2-macroglobulin	136	42
4th	albumin	10	22

ing this protein maximum will be continued The first protein fraction which contains pure γ-globulin, carries practically no FAA

Besides having demonstrated a new FAA carrier protein in the serum – the α_2-macroglobulin – we have in the present study been able to corroborate our earlier report [5], according to which one of the FAA carrier proteins in the serum is albumin In the present study, some 35% of the serum FAA was protein-bound

Discussion

Continuing our earlier studies of FAA carrier proteins [2, 3, 5–7] the present study revealed that serum contained at least 3 FAA carrier proteins It was reported previously that albumin carries FAA [5] The present study corroborated this finding Furthermore, the α_2 macroglobulin of serum carries a considerable amount of FAA In addition to these two identified proteins, either the transferrin and or a γ-globulin fraction carries serum FAA This part of our study is not yet completed and the findings will be published later

In the present study, in the same way as earlier [2], about one third of the total FAA is bound with serum proteins It seems that when the amount of total FAA in the serum decreases (e g in deficiency states or *as a result of other diseases) the proportion of bound FAA increases* [unpublished observation] This might mean that, as FAA stores are depleted, the FAA chains which are free in the serum are first drawn upon According to preliminary observations [7] pathological conditions produce changes also in the FAA fraction bound to serum proteins It might per

haps be assumed that the 3 carrier system of FAA in the proteins must have something to do with FAA kinetics Furthermore, FAA is firmly bound to proteins and withstands e g ammonium sulphate precipitation and re-dissolution almost without unbinding [7] In the nephrotic syndrome, measurable amounts of protein bound FAA is excreted in the urine [unpublished observation]

Not until the third carrier protein in the serum has also been verified can studies of FAA kinetics be started both in physiological conditions and under the influence of various diseases The first item covers primarily the binding capacity of each individual FAA carrier protein and the factors which in normal conditions apparently affect it For instance, the questions as to which proteins first bind the FAA introduced into the system and how the kinetics of the various proteins continue must be clarified In this connection attention must again be devoted to the protein bound FAA free FAA ratio in different circumstances It will be of interest to obtain information on how the binding of FAA is organized in the storage organs (e g liver)

Medical literature has fairly exhaustively discussed the total variations of blood FAA in various states and especially in different diseases Special emphasis has been attached to the influence of pregnancy on FAA stores It has been found that pregnancy produces an FAA deficiency The visible sign are lowered FAA levels in the serum But it has been reported recently that the influence of contraceptive hormone drugs on human FAA metabolism is similar [1, 8–12] and that anabolic hormones may raise the FAA level in serum and red cells [4] Now, once the different FAA carrier proteins are known this problem of pregnancy can also be reconsidered as soon as it is clear which of the different kinds of hormones affect the binding of FAA to human proteins Another extensive line of study is afforded by the question of how the FAA metabolism is affected by the diseases with a direct influence on protein synthesis We have shown preliminarily [7] that changes in megaloblastic conditions take place in the binding of FAA to proteins

References

1 Petrie I and Frey, J W Folsäuremangelanämie als Folge des langfristigen Gebrauchs peroraler kontrazeptiver Mittel Schweiz med Wschr 51 101 (1971)

[illegible]

haps be assumed that the 3 carrier system of FAA in the proteins must have something to do with FAA kinetics Furthermore, FAA is firmly bound to proteins and withstands, e g ammonium sulphate precipitation and re-dissolution almost without unbinding [7] In the nephrotic syndrome, measurable amounts of protein bound FAA is excreted in the urine [unpublished observation]

Not until the third carrier protein in the serum has also been verified can studies of FAA kinetics be started, both in physiological conditions and under the influence of various diseases The first item covers primarily the binding capacity of each individual FAA carrier protein, and the factors which in normal conditions apparently affect it For instance, the questions as to which proteins first bind the FAA introduced into the sys tem and how the kinetics of the various proteins continue, must be clarified In this connection, attention must again be devoted to the protein-bound FAA free FAA ratio in different circumstances It will be of interest to obtain information on how the binding of FAA is organized in the storage organs (e g liver)

Medical literature has fairly exhaustively discussed the total variations of blood FAA in various states and especially in different diseases Special emphasis has been attached to the influence of pregnancy on FAA stores It has been found that pregnancy produces an FAA deficiency The visible sign are lowered FAA levels in the serum But it has been reported recently that the influence of contraceptive hormone drugs on human FAA metabolism is similar [1, 8–12] and that anabolic hormones may raise the FAA level in serum and red cells [4] Now, once the different FAA carrier proteins are known, this problem of pregnancy can also be reconsidered, as soon as it is clear which of the different kinds of hormones affect the binding of FAA to human proteins Another extensive line of study is afforded by the question of how the FAA metabolism is affected by the diseases with a direct influence on protein synthesis We have shown preliminarily [7] that changes in megaloblastic conditions take place in the binding of FAA to proteins

References

1 BUHAC, I and FINN J W Folsäuremangelanämie als Folge des langfristigen Gebrauchs peroraler kontrazeptiver Mittel Schweiz med Wschr *51* 101 (1971)

2 MARKKANEN, T. Pteroylglutamic acid (PGA) activity of serum in gel filtration. Life Sci 7 887 (1968)
3 MARKKANEN, T. and PELTOLA, O. Binding of folic acid activity by body fluids. Acta haemat., Basel 43 272 (1970)
4 MARKKANEN, T., MAKELÄ, M., and RUOTSI, A. Folate activity of blood during artificial anabolism. Int. J. Vit. Nutr. Res. 40 73 (1970)
5 MARKKANEN, T. and PELTOLA, O. Carrier proteins of folic acid activity in human serum. Acta heamat., Basel 45 106 (1971)
6 MARKKANEN, T. and HIMANEN, P. Pteroylglutamic acid in cerebrospinal fluid. Int. J. Vit. Nutr. Res. 41 79 (1971)
7 MARKKANEN, T., PELTOLA, O. and HIMANEN, P. Metaoblic aspects of serum PGA in megaloblastic states. Int. J. Vit. Nutr. Res. 41 457 (1971)
8 PATON, A. Oral contraceptives and folate deficiency. Lancet I 418 (1969)
9 RASER, J. F., FARQUET, J. J., and PETTIT, J. Megaloblastic anemia due to folic acid deficiency in a young woman on oral contraceptives. Acta haemat., Basel 45 319 (1971)
10 SHOJANIA, A. M., HORNADY, G., and BARNES, P. H. Oral contraceptives and serum folate level. Lancet I 1376 (1968)
11 SHOJANIA, A. M., HORNADY, G., and BARNES, P. H. Oral contraceptives and folate metabolism. Lancet I 886 (1969)
12 STREIFF, R. R. Folate deficiency and oral contraceptives. J. amer. med. Ass. 214 105 (1970)

Authors' address: Associate Prof. T. MARKKANEN, M.D., R. L. PAJULA, M.Sc., Docent S. VIRTANEN, M.D., and M.D. P. HIMANEN, Vanha Littoistentie 40, 20520 Turku 52 (Finland)

Acta Haemat. 48 151–157 (1972)

Spezifische Cholinesterase im intakten Knochenmark

M. GOOTZ und G. LASSMANN

Neurologisches Institut der Universität
(Vorstand Prof. Dr. F. SEITELBERGER), Wien

Abstract. For the first time the distribution of specific cholinesterases in cells of the intact bone marrow (megakaryocytes and precursor cells) of the penis bone of rats and mice is presented. The results are compared with findings in particles of bone marrow already published.

Key Words
Cholinesterase in bone marrow
Histochemistry
Megakaryocytes
Rat bone marrow
Thrombocyte formation

Über das Vorkommen von spezifischer Cholinesterase in Megakaryozyten bei Ratten liegt eine Reihe von Mitteilungen vor [2, 5]. Erst in jüngster Zeit wurde die feine Verteilung der Cholinesterase in den Zellen elektronenoptisch untersucht [3]. Jedoch fanden sich in der bisher vorliegenden Literatur keine Angaben über das Verhalten und die Verteilung der Cholinesterase in grösseren Anteilen des *intakten* Knochenmarkes.

Wir haben im Zuge von Innervationsstudien am Penis der weissen Ratte und Maus die spezifische Cholinesterase nach Karnowsky-Roots für die Darstellung nervöser Formationen eingesetzt. Der Penisknochen lässt sich relativ leicht in Serienschnitte zerlegen und hiebei das vor allem in seinem kolbig aufgetriebenen basalen Anteil enthaltene Knochenmark zur Ansicht bringen. Dabei konnte das Vorkommen und die Lokalisation der Cholinesterase im Markraum des Penisknochens dargestellt werden.

Material und Methode

Einer grösseren Anzahl weisser Mäuse und Ratten – die Tiere entstammten einer virusfreien Zucht des Hygieneinstitutes der Universität – wurde der Penis

2 MARKKANEN, T. Pteroylglutamic acid (PGA) activity of serum in gel filtration. Life Sci. 7 887 (1968)
3 MARKKANEN, T. and PELTOLA, O. Binding of folic acid activity by body fluids. Acta haemat., Basel 43 272 (1970)
4 MARKKANEN, T., MÄKELÄ, M., and RUOTSI, A. Folate activity of blood during artificial anabolism. Int. J. Vit. Nutr. Res. 40 73 (1970)
5 MARKKANEN, T. and PELTOLA, O. Carrier proteins of folic acid activity in human serum. Acta heamat., Basel 45 106 (1971).
6 MARKKANEN, T. and HIMANEN, P. Pteroylglutamic acid in cerebrospinal fluid. Int. J. Vit. Nutr. Res. 41 79 (1971)
7 MARKKANEN, T., PELTOLA, O., and HIMANEN, P. Metabolic aspects of serum PGA in megaloblastic states. Int. J. Vit. Nutr. Res. 41 457 (1971)
8 PATON, A. Oral contraceptives and folate deficiency. Lancet i 418 (1969)
9 RASER, J. E., FAROUTT, J. J., and PETITT, J. Megaloblastic anemia due to folic acid deficiency in a young woman on oral contraceptives. Acta haemat., Basel 45 319 (1971)
10 SHOJANIA, A. M., HORNADY, G., and BARNES, P. H. Oral contraceptives and serum folate level. Lancet i 1376 (1968)
11 SHOJANIA, A. M., HORNADY, G., and BARNES, P. H. Oral contraceptives and folate metabolism. Lancet i 886 (1969)
12 STREIFF, R. R. Folate deficiency and oral contraceptives. J. amer. med. 214 105 (1970)

Authors' address: Associate Prof. T. MARKKANEN, M.D., [illegible] cent S. VIRTANEN, M.D., and M.D. P. HIMANEN, Vanha [illegible] Lu 52 (Finland)

Resultate

Im langsgeschnittenen Knochenmarkraum finden sich herdformig angeordnete cholinesterasepositive Areale unterschiedlicher Grosse, die *durch ihre starke Reaktion besonders hervorstechen* (Abb 1) Bei stärkerer Vergrosserung zeigt sich, dass diese Areale einer grossen Zelle entsprechen Das Plasma dieser Zellen enthalt in unterschiedlicher Intensitat cholinesterasepositive Granula, deren Dichte in den Randpartien grosser ist als in den zentralen Abschnitten Diese grossen Zellen finden sich auch in einer Hamatoxylinfarbung einzeln oder zu zweit und sind durch ihren hellen Kern und ihr wenig tingiertes Protoplasma gekennzeichnet (Abb 2)

Sind solche Zellen angeschnitten, so zeigt sich eine mehr gleichformige Verteilung der spezifischen Reaktionsprodukte der Cholinesterase im Plasma In anderen Bezirken findet sich eine Vergrosserung des cholinesterasepositiven Areals, das nunmehr eine unterschiedliche Dichte aufweist (Abb 3), die als beginnende Formierung der Blutplattchen angesehen werden kann In anderen Abschnitten finden sich bereits in grosser Zahl neugebildete Blutplattchen, die eine unterschiedlich intensive Cholinesteraseaktivitat besitzen (Abb 3)

Diskussion

Die Tatsache, dass es sich in unserem Fall der cholinesterasepositiven Zellen im Knochenmark um Megakaryozyten handelt, ist durch die Untersuchung von ZAJICEK [6] beim Menschen und unterschiedlichen Tiergattungen erwiesen Der Autor konnte den Nachweis fuhren, dass bei Ratte und Maus im Gegensatz zum Menschen die Megakaryozyten eine stark positive Reaktion geben, die Erythrozyten hingegen eine schwache, ein Verhalten, das bei der Katze in extremer Weise vorliegt, wahrend das Meerschweinchen eine Mittelstellung hinsichtlich dieser Verteilung einnimmt Nach ZAJICEK [8] werden heute noch zwei Theorien über die Entstehung der *Thrombozyten verfochten Die eine*, dass sie direkt von Plasma der Megakaryozyten abstammen, die andere, dass sie aus den Kernen der Normoblasten entstehen Eigene Untersuchungen des Autors [7] bestatigten, dass das Plasma des Megakaryozyten in hoher Konzentration spezifische Cholinesterase enthalt und auch durch *Mikromanipulation abgetrennte Teile des Plasmas nach kurzer Inkubation*

eine starke cholinesterasepositive Reaktion geben. Zajicek [8] vertritt daher die Auffassung, dass es sich um einen Beweis dafür handelt, dass die Plättchen aus dem Plasma der Megakaryozyten gebildet werden, eine Ansicht, die inzwischen von Behnke [1] anhand elektronenoptischer Untersuchungen durch den Nachweis der Bindung der spezifischen Cholinesterase an die Plättchendemarkationsmembran bestätigt wurde.

In unserem Material fanden sich nun, und soweit bekannt, erstmals in einem intakten *in situ* belassenen Knochenmark beschrieben, grosse Zellen mit ausserordentlich positiver Reaktion des Plasmas, jedoch mit einer ausgeprägten zentralen Aufhellung, die, so scheint es, den sich abteilenden Arealen entspricht. Im weiteren Verlauf finden sich Anteile, die zahlreiche runde ausserordentlich cholinesterasepositive Elemente enthalten. Es handelt sich um Elemente mit unterschiedlich grossen, stark gefärbten Kernresten, deren Plasma noch immer in beträchtlichem Ausmass cholinesterasepositive *Granula enthält*. In wieder anderen Abschnitten sind diese Elemente nicht mehr gehäuft zu finden, sondern schon mehr diffus verstreut. Wenn sich auch lichtoptisch membranartig angeordnete dichtere Strukturen bei starker Vergrösserung im Plasma der cholinesterasepositiven Zellen nachweisen lassen (Abb. 2), so kann über eine Strukturbindung im Sinne der elektronenoptischen Ergebnisse *lichtoptisch* keine sichere Aussage gemacht werden. Unsere Untersuchungen ergänzen die vorliegenden Befunde und zeigen darüber hinaus die räumliche Verteilung der Megakaryozyten und vermutlich auch ihrer weniger differenzierten Vorstufen im intakten Knochenmark.

Zusammenfassung

Die Verteilung der spezifischen Cholinesterase in den Zellen des intakten Knochenmarkes (Penisknochen der Ratte und Maus) wird beschrieben. *Die Ergebnisse werden mit bereits vorliegenden Untersuchungen verglichen.*

Literatur

1. Behnke, O.: An electron microscope study of the megakaryocyte of the rat bone marrow. J. Ultrastruct. Res. 24: 412–433 (1968).
2. Kettis-Hakkers, H.: Histochemical enzyme studies on rat bone marrow. Blut *10*: 49–55 (1964).

3 Lautenschlager J Elektronenmikroskopische Untersuchungen zur Verteilung der Cholinesterasen im Megakaryocyten der Ratte Histochemie *26* 343–348 (1971)

4 Rogister, G et Gerebtzoff, M A Recherches histochimiques sur les acétylcholine et cholinestérases Acta anat *32* 39–50 (1958)

5 Volkmann A The origin of macrophages from bone marrow in the rat Brit J exp Path *46* 62–70 (1965)

6 Zajicek J Studies on the histogenesis of blood platelets Acta haemat, Basel *12* 238–244 (1954)

7 Zajicek, J Quantitative determination of acetylcholinesterase activity in individual megakaryocytes at various stages of maturation Experientia *12* 226–227 (1956)

8 Zajicek J Studies on the histogenesis of blood platelets and megakaryocytes Acta physiol scand *40* suppl., vol 138 (1957)

Adresse des Autors Doz Dr G Lassmann Neurologisches Institut der *Universität Wien* Schwarzspanierstrasse 17 *A-1090 Wien* (Österreich)

Acta Haemat. 48: 158–163 (1972)

DNA Synthesis During Human Eosinopoiesis

S. N. Wickramasinghe and B. Moffatt

MRC Experimental Haematology Unit, St. Mary's Hospital Medical School, University of London, London

Abstract. The *in vitro* ^{3}H-TdR labelling index of eosinophil myelocytes in normal human bone marrow was 16.1%. A similar value was observed in 3 patients with eosinophilia. Considerably higher labelling indices were seen in the eosinophil myelocytes of 1 folate deficient and 2 vitamin B_{12} deficient patients. Another 6 patients with megaloblastic haemopoiesis showed normal or near-normal eosinophil myelocyte labelling indices despite the presence of elevated labelling indices in the neutrophil promyelocyte-myelocyte pool in 5 of them. The results indicate some differences in the cytokinetic disturbances affecting neutrophil and eosinophil granulocytopoiesis.

Key Words
Autoradiography
DNA synthesis
Eosinophilia
Eosinopoiesis
Folate deficiency
Megaloblastic anaemias
Vitamin B_{12} deficiency

Some data are now available regarding the kinetics of eosinopoiesis in experimental animals [1, 4]. In contrast, there is still little information on the kinetics of normal and disturbed eosinopoiesis in man [3, 5]. The technique of *in vivo* labelling with tritiated thymidine (^{3}H-TdR), used for the detailed analysis of cell kinetics in animals, has a very limited application in man, because of the potential radiobiological hazard and the need for multiple marrow aspirations. The study of human haemopoiesis must, therefore, largely depend on the use of short-term marrow cultures.

We have studied the *in vitro* ^{3}H-TdR labelling index of proliferating eosinophils in patients with normal, increased and megaloblastic eosinopoiesis. The purpose of this investigation was to determine to what extent the kinetics of eosinophil proliferation can alter in disease.

Materials and Methods

Five haematologically normal cases, 7 patients with megaloblastic haemopoiesis due to vitamin B_{12} deficiency, 2 patients with folate deficiency and 3 patients (cases E C, I L, and M W) with a marked eosinophilia have been studied Case E C had a chronic eosinophilia of uncertain aetiology for 8 years His marrow cells showed a normal chromosome pattern The high eosinophil count in case I L was due to penicillin hypersensitivity and had persisted for at least 7 days prior to this study The eosinophilia in the 3rd patient (M W) was also of unknown aetiology and had lasted for 3 months

Marrow aspirates were incubated with 7–12 μCi ^{3}H TdR/ml (specific activity 5 000 Ci/M), in heparinised Hanks solution at 37 °C for 0 5 h The labelled marrow fragments were then smeared on glass slides and the smears stained by the May Grunwald Giemsa (MGG) method Photographic maps were made of selected areas of these slides and the position and maturity of eosinophils and their precursors recorded on them The MGG stain was then removed, the slide restained by the Feulgen method and cells in DNA synthesis detected by autoradiography as described previously [6] In the MGG stained smears the large eosinophilic granules occasionally obscured nuclear detail which was revealed after Feulgen staining These cells were reclassified during assessment of the autoradiographs

The proportion of marrow eosinophils was determined in each case studied by assessing a minimum of 2,500 nucleated marrow cells in MGG stained smears. Blood eosinophil counts were calculated from the total white cell count (determined on a Model S' Coulter counter) and the differential leucocyte count

Results

In normal bone marrow, DNA synthesis was not observed beyond the eosinophil myelocyte stage In the patients with an eosinophilia, an occasional large eosinophil metamyelocyte was labelled A few labelled large and giant eosinophil metamyelocytes were seen in all the megaloblastic marrows

Table I shows the ^{3}H-TdR labelling indices of eosinophil myelocytes in the patients studied The labelling indices in the 3 patients with an eosinophilia were all normal In contrast, the labelling index was elevated (35 0–48 1%) in 3 of the 9 megaloblastic patients Two of these were vitamin B_{12} deficient and the 3rd folate deficient The average labelling index in the megaloblastic group was therefore increased

The proportion of marrow eosinophils showed a wide variation in vitamin B_{12} or folate deficient marrows, being low, normal or high in individual cases (table I) The mean value for the entire megaloblastic

Table I. ^{3}H TdR labelling indices of eosinophil myelocytes

Diagnosis	Hb g/100 ml	Blood eosinophils per µl	Marrow eosinophils, %[1]		Labelling index, %	
			total[2]	myelocytes	mean	SD
Normal (5 cases)	12.5–17.1	170 (<1–500)	3.2 (3.0–3.5)	1.4	16.1	2.4
*Polyarteritis nodosa (case E. C.)	16.1	2000	15.4	5.1	17.2	2.1
Penicillin allergy (case I. L.)	14.8	2000	26.0	11.3	15.1	3.3
*Polyarteritis nodosa (case M. W.)	13.9	2400	10.0	3.3	18.4	2.5
Megaloblastic (9 cases)	5.5–13.3	98 (<1–325)	3.6 (2.0–5.9)	1.6	27.5 (15.1–48.1)	1.9

[1] Expressed as a percentage of all nucleated marrow cells.
[2] Includes eosinophil myelocytes, metamyelocytes and polymorphs.

group was normal. In the 3 megaloblastic patients with an elevated eosinophil myelocyte labelling index, the average value for the percentage of all types of marrow eosinophils was 4.4% (2.3% eosinophil myelocytes).

Discussion

The labelling index of eosinophil myelocytes in normal marrow was 16.1%. This figure is lower than the corresponding value of 30% for the neutrophil ... using the same technique [7]. During previous combined quan... tochemical and autoradiographic studies of human bone marr... ity of unlabelled eosinophil myelocytes encoun... ed had d... contents, ind... g that they are either G_1 ... mitotic, ... G_0 (out-of-c... ... (unpublished observa... Normal ... amyeloc... t incorporate ^{3}H-TdR ... g that ... not div... ... egaloblastic marrows ... nt c... ... loc... ... DNA synthesis but

whether these cells go on to divide successfully is uncertain Similarly, an occasional labelled large eosinophil metamyelocyte was seen in patients with an eosinophilia.

Increased eosinopoiesis may result from one or both of 2 mechanisms (1) an increased rate of differentiation of stem cells along the eosinophilic pathway and (2) a shortening of interphase (perhaps by a reduction in the long G_1 period) resulting in an increased number of cell divisions during the proliferative phase of eosinopoiesis If a proportion of normal eosinophil myelocytes are in fact in G_0, then an increase in the growth fraction would also lead to increased eosinopoiesis From a detailed study of a single patient with intense eosinophilia, using ^{3}H TdR as an *in vivo* label, STRYCKMANS *et al* [5] found no evidence of a shortening of interphase in the proliferating eosinophils and concluded that increased differentiation of stem cells was largely responsible for the eosinophilia. This view is supported by the finding of a normal labelling index in the eosinophil myelocytes of the three patients with eosinophilia in the present study (table I) In contrast to the situation during an eosinophilia, the labelling index of the neutrophil promyelocyte-myelocyte pool is elevated during a reactive neutrophil leucocytosis indicating either a shortening of interphase or an increase in the growth fraction in these cells [7]

The normal eosinophil myelocyte labelling index in patients with an eosinophilia does not necessarily imply normal cell proliferation In the unlikely event of an identical fractional decrease in the duration of both interphase and the DNA synthesis (S) period, the labelling index will be unaltered However, in other cell renewal systems where *in vivo* studies indicate a shortening of both interphase and the S period, there is a relatively greater reduction in interphase which would lead to an increased labelling index [2, 4]

In the three patients with megaloblastic haemopoiesis and an elevated eosinophil labelling index, the proportion of eosinophil myelocytes in the bone marrow, expressed as a percentage of all nucleated marrow cells, was increased Because of the increased cellularity of the marrow fragments in these patients and the likelihood of an expansion of the total volume of haemopoietic marrow, the increase in the total number of eosinophil myelocytes would be greater than that suggested by the slight increase in the proportion of these cells in the marrow As the kinetics of eosinophil myelocyte proliferation do not appear to alter during increased human eosinopoiesis, the high labelling indices in these three

megaloblastic patients must be attributed to a prolongation of DNA synthesis rather than a shortening of interphase or an increase in the growth fraction. Provided that the blood eosinophil turnover rate is not increased, the combination of a marrow eosinophilia and impairment of DNA synthesis suggests the presence of some degree of ineffective eosinopoiesis in these patients. Intramedullary cell death and disturbed DNA synthesis have also been described in megaloblastic erythropoiesis and neutrophil granulocytopoiesis [8].

In 6 of the megaloblastic patients (5 vitamin B_{12} deficient and 1 folate deficient), the labelling indices of the eosinophil myelocytes were normal. It, therefore, appears that DNA synthesis during eosinopoiesis may be unaffected or minimally affected in some vitamin B_{12} or folate deficient patients and grossly impaired in others. A similar situation has been reported in megaloblastic erythropoiesis where the cell cycle abnormalities were either absent or undetectable in patients with a mild anaemia [6]. The five vitamin B_{12} deficient patients with normal labelling indices in the eosinophil myelocytes showed increased labelling indices (48.0–65.6%) in the neutrophil promyelocyte-myelocyte pool. The folate deficient patient with a normal eosinophil myelocyte labelling index had a labelling index of 36.5% in the promyelocyte-myelocyte pool. The apparent difference in the reaction of the eosinophil and neutrophil series to vitamin B_{12} deficiency may be related to differences in the proliferative kinetics of stressed eosinopoiesis and neutrophil granulocytopoiesis. Thus, the increased number of myelocyte divisions which appear to occur during stressed neutrophil myelopoiesis [7] but not during stressed eosinopoiesis might make the neutrophil series more vulnerable. Alternatively, the neutrophil series may have a special metabolic need and higher requirement for vitamin B_{12}. Recent autoradiographic studies of the uptake of vitamin B_{12} in short-term marrow cultures showed no autoradiographic grains over eosinophils and their precursors at a time when some neutrophil myelocytes, metamyelocytes and polymorphs were labelled [9].

References

1 ANDERSEN, V. and BRO-RASMUSSEN, F.: Autoradiographic studies of the kinetics of eosinophils. Ser. Haematol. *1/4*: 33 (1968).

2 HANNA, I. R. A.; TARBUTT, R. G., and LAMERTON, L. F.: Shortening of the cell-cycle time of erythroid precursors in response to anaemia. Brit. J. Haemat. *16*: 381 (1969).

3 HERION J C GLASSER, R. M WALKER, R. I, and PALMER, J G Eosinophil kinetics in two patients with eosinophilia. Blood *36* 361 (1970)
4 SPRY C. J F Mechanism of eosinophilia. V Kinetics of normal and accelerated eosinopoesis Cell Tissue Kinet. *4* 351 (1971)
5 STRYCKMANS P A. CRONKITE, E. P GREENBERG M L., and SCHIFFER, L. M Kinetics of eosinophil leukocyte proliferation in man Proc. 12th Congr Int. Soc. Haematology New York (1968)
6 WICKRAMASINGHE, S N COOPER, E. H., and CHALMERS, D G A study of erythropoiesis by combined morphologic, quantitative cytochemical and autoradiographic methods Normal human bone marrow, vitamin B_{12} deficiency and iron deficiency anemia. Blood *31* 304 (1968)
7 WICKRAMASINGHE S N and MOFFATT B Observations on cell proliferation in human myelocytes Acta haemat., Basel *46* 193 (1971)
8 WICKRAMASINGHE, S N Kinetics and morphology of haemopoiesis in pernicious anaemia. Brit. J Haemat. *22* 111 (1972)
9 WICKRAMASINGHE, S N and CARMEL, R. Uptake of ^{57}Co-vitamin B_{12} by human haemopoietic cells *in vitro* Brit. J Haemat. *23* 307 (1972)

Authors address. Dr S N WICKRAMASINGHE and Miss B MOFFATT Department of Haematology St. Mary s Hospital Medical School *London W 2* (England)

Acta Haemat. 48 164–175 (1972)

The Effect of Persantin® on the Phosphate Compounds in Erythrocytes During Blood Conservation

B. ZACHARA

Department of Physiological Chemistry (Dir. Doc. Dr. M. KAŃSKI), Academy of Medicine, Łódź

Abstract. The content of phosphate compounds in blood conserved at 2–4 °C in ACD, ACD with adenosine and ACD with adenosine and persantin has been investigated. The decomposition of high-energy phosphate compounds (mainly ATP, GTP and 2,3 DPG) was much slower in the solution containing adenosine with persantin than in two other media during the 6-week period of blood conservation. The clinical importance of the application of persantin for the prolongation of blood conservation is discussed.

Key Words
Blood conservation
Erythrocyte phosphates
Erythrocyte storage
Persantin®
Phosphate metabolism

In the normal human erythrocytes about $^{1}/_{2}$ of the acid soluble phosphate is represented by 2,3-diphosphoglycerate (DPG) and $^{1}/_{4}$ by adenosine triphosphate (ATP) [42]. It is very important for the longer survival of erythrocytes after transfusion to keep the content of these compounds at a suitably high level during the conservation of blood [1, 2, 19, 35, 38, 39]. It has been stated that there is a close correlation between the concentration of DPG and ATP and the dissociation of oxyhemoglobine [1, 3, 9, 10, 16, 18]. The red blood cells which have lost a considerable amount of these compounds during conservation bind the oxygen more avidly and, hence, release it less readily in the tissues [12].

The DPG concentration in the intact erythrocytes is about 4 times higher than that of ATP. The variations of the DPG content in the physiological and pathological states are also higher than those of ATP [22]. During the conservation of blood in the ACD solution at 4 °C the red blood cells lose almost completely their DPG after a fortnight, whereas the ATP decomposes much slower and it can be found in 43–62% of the initial amount even after 20 days of storage [7, 11, 13, 16, 17, 24]. Adenine, adenosine, inosine, inorganic phosphate and other com-

pounds have been added to the blood conserved in the ACD solution in order to maintain the proper level of these biologically important compounds for a longer period of time The addition of adenine makes it possible to get more than 70% survival of erythrocytes after 5–6 weeks of conservation [1 20 37–39] Inosine and adenosine which penetrate easily inside the erythrocytes [41] in combination with other compounds, exert the even more favourable effect [1 4, 8, 17 33] Erythrocytes contain the active phosphorylase of purine nucleosides splitting inosine and adenosine after previous deamination [41] Phosphorolysis of inosine leads to the formation of hypoxantine and ribose phosphate the latter being transformed into fructose-6-phosphate and triose phosphate in the pentose phosphate cycle giving energy in further transformations in the glycolytic pathway [17 21] It is considered that a certain part of adenosine added to the conserved blood may undergo phosphorolysis yielding not only ribose phosphate but also adenine which is incorporated into the adenine nucleotides [8 14] According to the more recent data the direct phosphorolysis of adenosine to adenosine monophosphate (AMP) with the participation of the adenosine kinase seems to be more probable [8 29 40 42]

GIBSON and LIONETTI [24, 31] have found that the addition of persantin – the inhibitor of adenosine deaminase – to the blood conserved in ACD with adenosine added, leads not only to the slower drop in the content of ATP during the conservation but is the cause of the considerable increase of the ATP content in the initial period of the blood storage

The aim of this work was to observe the content of nucleotides and other important phosphate compounds in the red blood cells conserved in ACD with adenosine and in ACD with adenosine and persantin added

Materials and Methods

Preparation of the conservating solutions ACD solution (formula B) was used for the conservation of blood The 25 ml portions of this solution were placed in 3 125-ml glass bottles 81 73 mg portions of adenosine (Reanal Hungary) were added to 2 bottles containing ACD solution (ACD-A) A sample of 6.3 mg of persantin (Persantin® Nr F 2624 Boehringer Sohn, Ingelheim, 2,6-b s [diaethanoloamino]-4 8-dipiperidinopyrimido-5 4-d pyrimidine) was dissolved in 1.5 ml of 95% ethanol and added to one of the prepared ACD-A solutions (ACD-A P) All solutions were autoclaved.

Collection and preparation of blood Blood was supplied from the Blood Centre 300 ml of blood were taken from one blood donor (100-ml portions to each of the three prepared solutions) The ratio of blood to the conservant was 4 1 Adenosine and persantin concentrations were 2 mM and 1×10^{-4} M respective-

ly. The collected blood was distributed aseptically in about 16-ml portions into the bottles containing 1 ml of 0.9% NaCl solution. This was done in a chamber sterilized with a bacteriological lamp. No bacteriological contamination was observed during the conservation period. The conservation was carried out at +2 to +4 °C.

Methods. Three kinds of blood (ACD, ACD-A and ACD-A-P) were examined always simultaneously. Blood was centrifuged, plasma and the buffy coat were removed and the remaining packed red cells were resuspended in 4 vol. of 0.9% NaCl solution and centrifuged. The supernatant fluid was removed, and after addition of 1 vol. of 0.9% NaCl solution the microhematocrites were determined. 3 ml of the packed cells were taken for the determination. The acid soluble phosphate compounds were obtained according to BARTLETT [5]. After the threefold extraction with trichloroacetic acid, the acid was removed with ether, the extract was neutralized and frozen at −25 °C. Separation of the phosphate compounds was carried out within 1–4 days. After thawing the extract was diluted always to the constant volume and double samples were taken for the determination of the total phosphate. Phosphate compounds were separated chromatographically using Dowex 1X8 formate form, 200–400 mesh (Serva, Entwicklungslabor, Heidelberg) in a glass column 0.8×15 cm. Formic acid and ammonium formate solutions [32] were used for the elution at a rate of about 1 ml/min and at a slight overpressure [26]. 3- to 3.5 ml fractions were collected and their optical density was measured at 260 nm (Spectromom 202, Hungary). The determination of phosphate [6] was limited in most cases to the inorganic phosphate (P_i) and total phosphate (TP) in a DPG peak. To identify the compounds in few separations TP was determined in all fractions. Samples showing the highest optical density at 260 nm of particular peaks were passed through the active charcoal (HGK 027, Takeda Pharmaceutical Co., Japan) in order to adsorb the nucleotides which were then eluted with ethanol and ammonia solutions. The eluates were lyophilized, the dry residue dissolved in water, pH adjusted to 2, 7 or 11, and the spectrum measured spectrophotometrically. Nucleotides were identified by the absorption ratios 250/260 and 280/260 nm, and by the molar ratios of nucleotide bases to phosphate. Beyond this, reference experiments were carried out in which standard samples of nucleotides occuring in blood cells and DPG were separated in a column.

Results

The content of adenine nucleotides in the blood conserved in ACD, ACD-A and ACD-A-P. The initial content of ATP was almost identical in the red blood cells conserved in three different media (fig. 1). After 3 weeks of conservation in ACD, the ATP level lowered to about 60% of the initial amount. During the whole 6-week period of conservation, the drop in the content of ATP was nearly linear and proportional to the time. In the 6th week there was still about 1/4 of the initial amount in the red cells. The content of ATP in the blood conserved in ACD-A was similar in

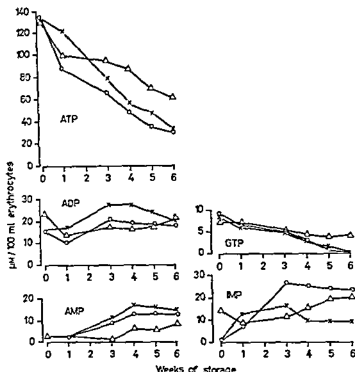


Fig 1 Changes in nucleotide content in erythrocytes of blood stored during a 6-week period at 2-4 °C in various media. ACD (X) ACD-A (O) ACD-A P (△). The values are expressed in micromoles of nucleotide per 100 ml packed cells.

the final period but during the 1st week of conservation the much faster drop was observed About 20% drop in the content of ATP was also observed after the 1st week in the medium containing persantin Later, the decomposition was much slower and in the 3rd week the red cells contained ³/₄ and after 6 weeks about 50% of the initial amount – 2 times more than in ACD and ACD-A solutions

The level of adenosine diphosphate (ADP) in the blood conserved in ACD was slightly elevated after 7 days, reaching a maximum value between the 3rd and 4th week (70% higher in comparison with the initial content, fig 1) Later, a slow drop was observed and after 42 days the content was still about 12% higher than in the initial period. A distinct

drop in the amount of ADP took place in 2 other media (ACD A and ACD A-P) after 7 days In the 21st day the content increased in both media and was more significant in ACD A solution These amounts were, however, much lower than in the ACD blood Beginning from the 3rd week a progressive drop in ADP was observed in the blood with ad enosine added, reaching in the 42nd day the level a little higher than the initial A slow increase of the ADP content in the further period of conservation was observed in the medium containing persantin In the last medium the content of AMP remained on the same, unchanged low level up to the 3rd week *After that time it increased gradually, reaching in* the 42nd day the amount 3 times higher than that at the beginning (fig 1) A rather significant increase of the amount of this nucleotide occured in the medium with ACD and ACD A from the 1st to 4th week (in the 28th day it was 7 and 5 times higher, respectively, as compared with the 'zero day') In the next weeks there was a small drop in the ACD blood, but the level was unchanged in ACD A

Other free nucleotides in the red blood cells The product of deamination of AMP – inosine monophosphate, (IMP) – is strictly connected with the metabolism of adenine nucleotides Its presence was not observed at the beginning of the investigation neither in the blood with ACD, nor with ACD and adenosine Striking is a relatively high level of this compound (14 7 μM/100 ml of erythrocytes) in the first hours of conservation with adenosine and persantin (fig 1) Its presence was also observed after 1 week in the blood of other media Within the next 5 weeks its level in ACD, except slight variations, remained the same It reached the highest value (26 5 μM/100 ml of erythrocytes) in ACD A blood at the end of the 3rd week A slow drop was observed during the next 3 weeks During the 1st week there was a slight decrease of its amount in the ACD A P blood and in the next weeks a progressive in crease had a linear character

The drop in the content of guanosine triphosphate (GTP) during the 6-week period of conservation was similar to that of ATP It was the slowest in the ACD A P blood reaching in the 42nd day up to about 60% of the initial value A complete decomposition of GTP was observed in 2 other media at the end of the 6th week The decomposition of coenzymes nicotinamide adenine dinucleotide (NAD) and its phos phate (NADP) was relatively small in all the three media The drop in the content of NAD and NADP at the end of the 6th week was about 17 and 20% respectively

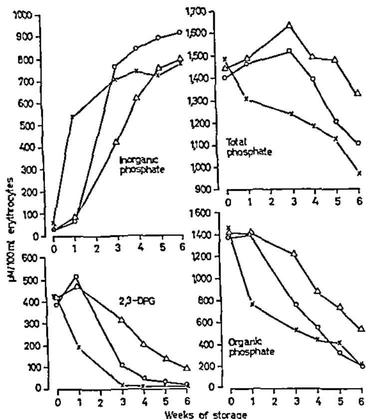


Fig 2 Changes in inorganic phosphate, 2,3-diphosphoglycerate(2,3-DPG) total phosphate and organic phosphate in erythrocytes Blood was stored during a 6-week period at 2–4 °C in ACD (X) ACD A (O) and ACD-A P (△) medium The values are expressed in micromoles of 2 3-DPG and above mentioned phos phates per 100 ml of packed cells.

2,3 Diphosphoglycerate, total phosphate, organic and inorganic phosphate The content of DPG, TP, organic and inorganic phosphate (P_i) are presented in figure 2 The decomposition of DPG in the blood conserved in ACD was very fast. It was 50% lower after 1 week and beginning from the 3rd week only traces of the DPG amount could be found The level of DPG in 2 other media increased after 1 week exceeding the

initial value in ACD-A and ACD-A-P by about 34 and 13%, respectively. This period was followed by a decrease of DPG level which was more distinctly marked in the ACD-A blood. The drop was linear and much slower in the ACD-A-P solution: In the 42nd day there was still above 20% of the initial amount. Curves representing organic phosphate (the difference between the total and P_i) in the particular periods are similar to those of DPG. The fastest drop during the initial period was characteristic of blood conserved in ACD, the slowest in ACD-A-P. At the end of the 6th week the erythrocytes with ACD and ACD-A contained above 10% and those with ACD-A-P nearly 40% of the initial amount. Curves relating to total phosphate (fig. 2) for each of the three investigated media are also different. The drop was significant and proportional to the time in the erythrocytes with ACD. In the erythrocytes with ACD-A and ACD-A-P the increase in the first weeks exceeded the initial level. The decrease was observed from the 21st day, reaching in the 42nd day about 80% in ACD-A and 90% in ACD-A-P blood of the initial content.

The decomposition of the organic phosphate compounds is followed by the accumulation of P_i in the red blood cells. In the 1st week it was particularly significant in blood with ACD, being much lower in 2 other media. In the preservative solution containing persantin the increase of P_i level was the slowest and almost linear within the 6-week period of conservation. On the 42nd day, however, the increase was almost the same in all the three media.

Discussion

It has been stated in a previous paper [43] and in the experiments of other authors [7, 17, 33] that blood conserved in ACD loses rather rapidly the high-energy phosphate compounds. The DPG undergoes decomposition most rapidly. NAKAO *et al.* [34] found that the red blood cells lose 30% of DPG already after 24 h of conservation. In that time the ATP level undergoes no greater changes. It is generally known that as long as the red blood cell contains DPG the drop in ATP is slow. The drop in DPG to very low values is followed by a rapid decrease of the ATP content [7]. These observations are also confirmed by this work. The drop in the content of ATP is accompanied by the accumulation of the products of its decomposition: ADP, AMP and IMP in the red

blood cell The content of these compounds at the end of the 6th week of conservation is higher than at the beginning. The sum of the adenine nucleotides (ATP, ADP, AMP) and IMP, which is strictly connected with AMP metabolism, decreases distinctly already in the 3rd week of conservation. This is probably due to the continuous deamination of AMP and decomposition of the product to inosine and P_i The increase of P_i inside the red blood cell, though it reflects the decomposition of the organic phosphate compunds, is usually lower than the amount of the liberated P_i This is connected with the accomplishment of the saturation point in the medium inside the red blood cell [7] and penetration of P_i through the membrane of erythrocyte into surrounding medium [25]

Enrichment of the conservating medium with adenosine exerts a great influence upon the maintenance of DPG and ATP contents on a proper level [33] CHANUTIN [17] found that the DPG content increases above the initial level at the beginning of the conservation period Similar results were obtained in this work, though the used adenosine concentration was lower that the optimal The increase of DPG level is probably connected with the appearance of a new substrate – ribose phosphate, yielded in the way of phosphorolysis of the nucleoside [14, 17] A more significant fall in ATP was, however, observed in comparison with the blood conserved in ACD At the end of the 6th week of conservation the amount of ATP in both media was the same GIBSON and LIONETTI [24], who used the same concentration of adenosine, also observed a similar phenomenon.

The presence of adenosine and persantin in the blood conserved in ACD exerts a positive effect on the maintenance of the proper level of ATP [24] and also, according to this experiment, of both GTP and DPG During the whole period of observation the amount of the organic phosphate compounds was on a much higher level than in the two other media GERLACH *et al.* [23] found that persantin reduces the efflux of the P_i from the erythrocytes. A much slower increase of P_i content in the medium containing persantin was observed in this work. This is a proof of a slower decomposition of the organic phosphate compounds in erythrocytes The increase of the amount of IMP during the conservation confirms the results obtained by LIAN *et al* [30], who found no inhibitory effect of persantin upon the AMP deaminase from the human erythrocytes The same is true for adenine, adenosine and inosine which do not inhibit this enzyme. 2,3-DPG and P_i exert a competitive inhibi-

tory effect ($K_i \approx 16$ and 7.5 mM, respectively) but ATP diverts it. Proper intracellular concentrations of DPG and ATP inhibit the action of AMP deaminase in about 25%.

BUNAG *et al.* [15] proved that the action of adenosine deaminase is inhibited by persantin only in the full human blood and is not affected in hemolysates. They also succeeded in showing that persantin penetrates easily inside the erythrocytes and that, after the repeated washing of the red blood cells in the physiological NaCl solution, about 20% of this compound remains inside the erythrocytes, produce an 'adenosine sparing effect'. The optimal concentration of this inhibitor, on account of the high-energy compounds, is from 1×10^{-4} to 4×10^{-4} M [24]. Higher concentrations cannot be applied because of low solubility in water [15]. We observed in the last two weeks of conservation the increasing haemolysis of erythrocytes in the ACD and ACD-A media. In the presence of persantin haemolysis occured not earlier than at the end of the 6th week and was much less marked. A similar phenomenon was observed by PHILIPP *et al.* [36]. In their experiments a spontaneous haemolysis in the presence of persantin after a 6-week period of conservation was by 36% lower than the blank. The osmotic resistance in a 0.6 percent NaCl was approximately about 50% higher in the period from 20 to 56 days of conservation [36].

The therapeutic properties of persantin are well known to clinicists. No toxic injuries of organs [27] were observed in the experimental investigations carried out with animals which were submitted to the action of high doses of this drug within several months. The use of i.v. doses from 30 to 40 mg [28] is recommended in some heart diseases of men. A drop in the level of this drug in the blood occurs 15 min after the injection and it reaches a half of its initial value after 25 min [24]. If the optimal concentration (1×10^{-4} M) is used for the conservation 500 ml of blood will contain about 25 mg of the drug. Assuming that the transfusion of this amount of blood takes about 60 min, the penetration of the drug from blood into tissues and body fluids and even excretion occurs during the transfusion.

Further experiments will show whether the transfusion of blood containing the above given concentrations of persantin will be harmless to the recipient. In the cases when transfusion of the packed red blood cells is necessary, the amount of drug introduced with the erythrocytes is much lower than that used for conservation of full blood. KÜBLER *et al.* [25] found namely that persantin combines much stronger with the plas

ma than with the erythrocytes The coefficient of distribution of the drug between plasma and erythrocytes of men is 6 53

Prolongation of the period of blood storage for a longer period of time in the presence of persantin may be of a great importance in blood centres which have no possibility to store blood at low temperatures

Acknowledgements I wish to express my appreciation to C. H. Boehringer Sohn, Ingelheim, Germany for the kindly supplied of Persantin used in these ex periments, and to Prof SHIGEKI MINAKAMI, Department of Physical Chemistry, Faculty of Medicine, University of Tokio Japan for his providing of charcoal I would also like to thank Prof WANDA LEYKO, Institute of Biophysics, University of Łódź, Poland for her critical remarks and to Mrs BARBARA KACZMAREK for her skilled technical assistance

References

1 AKERBLOM, O, DE VERDIER, C. H., ERIKSSON A., GARBY, L., and HÖGMAN, C. Late addition of inosine to stored ACD-adenine blood. Effect on erythrocyte viability and metabolism. Folia haemat. *91* 142–150 (1969)

2 AKERBLOM, O, DE VERDIER, C. H., FINNSON M, GARBY, L., HÖGMAN, C. F, and JOHANSSON, S G O Further studies on the effect of adenine in blood preservation Transfusion *7* 1–9 (1967)

3 AKERBLOM, O, DE VERDIER, C H., GARBY, L., and HÖGMAN, C. Restoration of defective oxygen transport function of stored red blood cell by addition of inosine Scand. J clin. Lab Invest. *21* 245–248 (1968)

4 AKERBLOM, O and ERICSON, A Effect of inosine, pyruvate and inorganic phosphate on the 2,3-DPG level in fresh and stored erythrocytes 6th Int. Berlin Symp on Structure and Function of Erythrocytes, Berlin 1970

5 BARTLETT, G R. Human red cell glycolytic intermediates J biol Chem *234* 449–458 (1959)

6 BARTLETT, G R Phosphorus assay in column chromatography J biol Chem. *234* 466–468 (1959)

7 BARTLETT, G R. and BARNET, H N Changes in the phosphate compounds of the human red cell during blood bank storage J clin. Invest. *39* 56–61 (1960)

8 BARTLETT, G R and BUCOLO G The metabolism of ribonucleoside by the human erythrocyte Biochim. biophys. Acta *156* 240–253 (1968)

9 BENESCH R. and BENESCH, R E. The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin Biochem biophys Res Commun. *26* 162–167 (1967)

10 BENESCH, R. E. and BENESCH, R Interaction of red cell organic phosphates with hemoglobin Forsvarsmedicin Stockh *5* 154–158 (1969)

11 BEUTLER, E., MEUL, A., and WOOD, L. Depletion and regeneration of 2,3-di phosphoglyceric acid in stored red blood cells Transfusion *9* 109–114 (1969)

12 BEUTLER, E. and WOOD L. The *in vivo* regeneration of red cell 2,3-diphosphoglyceric acid (DPG) after transfusion of stored blood J Lab clin. Med *74* 300–304 (1969)

13 BISHOP, C. Changes in the nucleotides of stored or incubated blood Transfusion *1* 349–354 (1961)

14 BISHOP C. Overall red cell metabolism, in BISHOP and SURGENOR The red blood cell, pp 147–188 (Academic Press, New York 1964)

15 BUNAG R D, DOUGLAS, C. R IMAI, S., and BERNE, R. M. Influence of a pyrimidopyrimidine derivative on deamination of adenosine by blood Circulat. Res *15* 83–88 (1964)

16 BUNN H F, MAY, M H. KOCHOLATY, W F., and SHIELDS, C. E Hemoglobin function in stored blood J clin Invest *48* 311–321 (1969)

17 CHANUTIN, A Effect of storage blood in ACD adenine-inorganic phosphate plus nucleosides on metabolic intermediates of human red cells Transfusion *7* 409–419 (1967)

18 CHANUTIN, A and CURNISH R. R. Effect of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes Arch Biochem Biophys. *121* 96–102 (1967)

19 DERN, R J, BREWER, G J., and WIORKOWSKI, J J Studies on the preservation of human blood II The relationship of erythrocyte adenosine triphosphate levels and other *in vitro* measures to red cell storageability J Lab clin Med *69* 968–978 (1967)

20 DE VERDIER, C. H, GARBY, L., HJELM, M., and HÖGMAN, C. Adenine in blood preservation Posttransfusion viability and biochemical changes Transfusion *4* 331–338 (1964)

21 GABRIO, B W, HENNESSEY, M, THOMASSON, J., and FINCH C. A Erythrocyte preservation IV The *in vitro* reversibility of the storage lesion J biol Chem. *215* 357–366 (1955)

22 GARBY, L., GERBER, G., and DE VERDIER, C. H Binding of phosphocompounds to haemoglobin Försvarsmedicin, Stockh *5* 163–166 (1969)

23 GERLACH E., DEUTICKE, B und DUHM, J. Phosphat Permeabilität und Phosphat Stoffwechsel menschlicher Erythrocyten und Möglichkeiten ihrer experimentellen Beeinflussung Pflügers Arch ges Physiol *280* 243–274 (1964)

24 GIBSON J G II and LIONETTI F J The effect of dipyridamole on the adenosine triphosphate level of stored human blood Transfusion *6* 427–437 (1966)

25 GLADER, B E. and OMACHI A Role of cellular P_i in P_i transport and metabolism in human red cells Biochim. biophys Acta *150* 524–527 (1968)

26 GREGER J and ZACHARA, B An apparatus for continuous gradient elution in ion exchange chromatography under pressure Biul WAM Łódź *11* 203–208 (1968)

27 KADATZ, R Die pharmakologischen Eigenschaften der neuen coronarerweiternden Substanz 2,6-Bis(diaethanol-amino)-4,8-dipiperidino-pyrimido(5,4-d)pyrimidin. Arzneimittelforsch. 9 39–45 (1959)

28 KÜBLER, W, BRETSCHNEIDER, H J und SPITCKERMANN P G. Die Bindung des Coronardilatators Dipyridamol im Plasma des Menschen und des Hundes sowie

ihre Bedeutung des Medikamentes beim Menschen. Klin Wschr 47 108–109 (1969)

29 LERNER, M H. and RUBINSTEIN, D The role of adenine and adenosine as pre cursors for adenine nucleotide synthesis by fresh and preserved human erythrocytes. Biochim. biophys Acta 224 301–310 (1970)

30 LIAN, C. J, GRAYSON, V., and HARKNESS, D R. Properties of AMP deaminase purified from human erythrocytes 13th Annu Meet. Amer Soc. Hemat., San Juan, Puerto Rico 1970

31 LIONETTI, F J and GIBSON, J G II The effect of dipyridamole on the adenosine triphosphate level of stored human blood. Proc. 11th Congr Int. Soc. Blood Transf., Sydney, 1966, in Bibl haemat., vol 29, pp 751–757 (Karger, Basel 1968)

32 MILLS, G C., BURGER, D O, SCHNEIDER, M., and LEVIN, W C. Ion exchange analysis of organic phosphate compounds of erythrocytes from individuals with neoplastic diseases. J Lab clin Med. 58 725–735 (1961)

33 MOLLISON, P L. and ROBINSON, M A Observation on the effects of purine nucleosides on red-cell preservation Brit. J Haemat. 5 331–343 (1959)

34 NAKAO, M, NAKAO, T, ARIMATSU, J., and YOSHIKAWA, H. A new preservative medium maintaining the level of adenosine triphosphate and the osmotic resist ance of erythrocyte Proc. Japan Acad. 36 43–47 (1960)

35 NAKAO K., WADA, T, KAMIYAMA, T, NAKAO, M., and NAGANO, K. A direct re lationship between adenosine triphosphate level and *in vivo* viability of eryth rocytes. Nature 194 877–878 (1962)

36 PHILIPP, G RUCKPAUL, K., BANASCHAK, H und JUNG, F Einfluss von Dipyri damol auf die Blutkonservierung Acta biol. med. germ 23 suppl K, pp 31–36 (1969)

37 SHIELDS, C.E., BUNN H F, LITWIN, S.D, REED, L.J., and DAUBER, L.G Clinical evaluation of transfused blood after long term storage in ACD with adenine Transfusion 9 246–250 (1969)

38 SIMON E. R. Adenine and purine nucleotides in human red cell preservation. A review Transfusion 7 395–400 (1967)

39 SIMON, E. R, CHAPMAN, R. G., and FINCH, C. A. Adenine in red cell preser vation. J clin Invest 41 351–359 (1962)

40 SUGITA, Y and SIMON, E. R. The mechanism of action of adenine in red cell preservation. J clin Invest. 44 629–642 (1965)

41 VAN BELLE, H.. Uptake and deamination of adenosine by blood Species differ ences, effect of pH, ions, temperature and metabolic inhibitors. Biochim. biophys Acta 192 124–132 (1969)

42 YOSHIKAWA, H. and NAKAO M Biochemistry of red cells stored in liquid me dia. Ser Haematol 10 48–60 (1966)

43 ZACHARA, B, DOMICZ, U, KAŃSKI, M., and LEYKO W Phosphate esters in erythrocytes frozen in DMSO Clin chim Acta 18 127–132 (1967)

Author's address Dr B ZACHARA, Department of Physiological Chemistry, Academy of Medicine Pl 9 Maja 1 *Łódź* (Poland)

Acta Haemat 48 176–182 (1972)

Platelet Function Studies in Factor V Deficiency

RUTH ANDREA SEELER and M ZIAD SHWIAKI

Hektoen Institute of Medical Research, Cook County Hospital
Division of Pediatrics, Chicago, Ill

Abstract Two unrelated black children (male and female) with a clinically mild hemorrhagic diathesis were found to be factor V deficient (<1 and 2.5%) One was diagnosed at one week of age, the other later in childhood Symptoms included umbilical hemorrhage, epistaxis, gastrointestinal bleeding and hemorrhage following oral mucous membrane lacerations The factor V level in the mother of one patient was borderline low while factor V levels in the parents of the other child were normal *Platelet function studies on both children were normal*

Key Words
Bleeding disorders
Blood coagulation
Factor V deficiency
Platelet function

Following the original description of factor V deficiency, parahemophilia, by OWREN [9] in 1947, 58 cases have been recorded in multiple ethnic groups, however, none stated the patients to be black [13] It is quite likely that many patients were not studied because of the mild nature of the hemorrhagic symptoms [13] In factor V deficiency the predominant symptom is epistaxis, a hemorrhagic manifestation usually associated with the platelet disorders The bleeding time, an accepted test of platelet function, was prolonged in approximately $^1/_3$ of the previously reported cases [13]

Factor V (labile factor, ac globulin) intimately bound to the platelet surface, was originally designated as platelet factor 1 [6] Platelets from patients with factor V deficiency are deficient in factor V activity, but when suspended in normal plasma adsorb factor V [1, 6, 7, 14] Platelet aggregation provides a method of studying factor V deficient platelets in factor V deficient plasma because aggregation is independent of clot formation

This paper reports 2 cases of factor V deficiency in unrelated black children The results of a variety of platelet function tests assess the role

of platelt factor V on platelet adhesion, aggregation and platelet factor 3 release

Case Reports

Case 1 The patient was born 3 2 69 at term to a gravida 1, para 1, 17 year old mother after an uneventful pregnancy and delivery The perinatal period was normal and she was discharged on the 3rd day The patient was readmitted 4 days later with a 5 hour history of bleeding from the umbilical stump Coagulation studies revealed that the factor V level was less than 1% (table I) Application of a dressing soaked with topical thrombin provided excellent hemostasis.

At 5 months of age, a laceration of the frenulum required the infusion of 10 ml kg of fresh frozen plasma (FFP). A second infusion was given empirically 24 h later ε Amino caproic acid (EACA), 100 mg kg every 4 h was administered concurrently Hematomas of the chest and extremities were first noted at 7 months of age A fingernail scratch wound of the ear bled for 20 min before the grandmother could stop it.

At 14 months, a severe diaper rash with multiple small abscesses developed and the patient became overtly septic The lesions enlarged to 6 by 8 cm and yielded 10-15 ml of seropurulent material on aspiration Coagulase positive, hemolytic *Staphylococcus aureus* was isolated in pure culture Therapy with oxacillin daily infusions of 10 ml kg of FFP and repeated aspirations resulted in resolution Because the abscess cavities were deep within the buttocks, aspiration was considered safer than incision and drainage She has had no additional pyogenic infections.

At 18 months of age she sustained a laceration of the lower lip Topical bovine thrombin initially controlled the bleeding however, the clot became dislodged and significant hemorrhage ensued The transfusion of 20 ml kg of fresh whole blood provided good hemostasis.

The child severed the tip of the right index finger and was treated elsewhere with wound debridement and suturing. Because there was no bleeding the grandmother did not bring the child for further therapy The wound, which extended completely across the finger pad healed uneventfully

At 25 months, a persistent oozing from cracked lips associated with an upper respiratory tract infection necessitated a single infusion of 10 ml kg of FFP The factor V level 60 h later was 4%. The next hemorrhagic episode was 2 years later (4½ years old) when she bit her tongue After 12 h of persistent oozing she was brought to the hospital A single infusion of 10 ml kg of FFP promptly stopped the hemorrhage EACA 100 mg kg dose every 6 h was given for 2 days

Routine immunizations have not produced any noticeable hematomas. The eruption of the deciduous teeth was uneventful

The family history was negative for epistaxis menorrhagia, *post partum* or dental hemorrhage surgical or infantile hemorrhagic deaths The father was not known to have a history of bleeding and was unfortunately unavailable for study There is no known *consanguinity*

Case 2 L. M., a 12-year-old black male (BD 2. 11 59) was well until 13 months of age when he began to have epistaxis usually controllable by pressure. At 5 years of age a 3 hour episode of epistaxis prompted investigation of the coagulation mechanism leading to the diagnosis of factor V deficiency. When the patient was 6 years old, an episode of gastrointestinal hemorrhage required transfusion. Radiographic studies of the upper and lower gastrointestinal failed to delineate the origin of the hemorrhage.

The child has done reasonably well with epistaxis controllable at home. He is extremely active and has sustained multiple lacerations and abrasions, all of which responded to local measures. A foot laceration required suturing but no replacement therapy. Bruises are constantly present, however routine immunizations were not associated with hematomas. Eruption and shedding of the deciduous teeth have not been associated with hemorrhage.

At 11 years of age, he sustained a fracture of the left clavicle. The overlying hematoma measured 5×6 in but no transfusions were necessary. The factor V level was found to be 2.5% (table I).

Family history revealed that the paternal grandmother was aware of easy bruising but had no hemorrhage following appendectomy, mastectomy, oophorectomy or delivery. There was no consanguinity. A sibling of the patient died in infancy of congenital hydrocephalus, two siblings are alive and well.

Coagulation Studies

Plastic syringes, pipettes and test tubes were utilized except disposable glass serology tubes for the whole blood clotting time and clot retraction. Using a 2 syringe technique the blood was drawn with a 21 guage scalp vein needle, anticoagulated in the ratio of 9:1 with 3.8% citrate and placed on melting ice. The samples were centrifuged in a refrigerated centrifuge for 15 min at 10,000 rpm.

First stage factors XII, XI, IX, VIII were assayed using congenitally deficient [illegible] as in a modif[illegible] of the PTT [16]. Reference plasma obtained from Hy[illegible] laboratories [illegible] normal plasma for factor XII were employed as [illegible] Commer[illegible] [illegible]plastin (Dade) and citrated normal plasma (Dade) [illegible] the p[illegible] [illegible] me control and to establish the standard curves [illegible] for [illegible], VII X II (PT [illegible] Factor V substrate was [illegible] method [illegible] et al. [1]5 [illegible] II deficient substrate was [illegible] binige[illegible] [illegible] (Dade) and normal ser[illegible] [illegible] was [illegible] deprothrombinized beef [illegible] plas[illegible] [illegible] Fibrometer

A [illegible]

[illegible] not chilled. Platelet [illegible] et al. [1]8 platelet [illegible] technique [illegible]

Platelet factor 3 (PF 3) was a modification of the method of HARDISTY and INGRAM [4] Briefly, the test is an incubation of platelet rich plasma (PRP) and platelet poor plasma (PPP) with kaolin for 20 min, which is then calcified The absence of plasma factor V makes it impossible to determine if PF 3 was released by factor V deficient platelets in V deficient plasma because the clotting time is prolonged in the absence of factor V Therefore, an additional 0 1 ml of normal or V deficient plasma was added immediately prior to calcification

Results

All parameters measured were normal except factor V levels, which was <1% in case 1 and 2 5% in case 2 (table I) Prothrombin times of PPP and PRP were similar

The platelets of our patients released PF-3 normally (table II) In the modification of PF-3 test, it may be reasoned that the added factor V

Table I

	Case 1	Case II
PTT (30–45 sec)	150	53–76
PT (10–11)	30 7 34 9	28 0–32 3
Clotting time (12 min)	20	17
Thrombin time (11–15 sec)	11 9	12 3
Fibrinogen (200–400 mg)	312	352
Factor assay %		
V	1	2 5
II	95–100	100
PP	75–88	96
VIII	114	150
IX	72	70
XI	108	74
XII	88	140
XIII	normal	normal
Inhibitor	negative	negative
Platelets × 10³	225–346	360,000
Clot retraction	good	good
Bleeding time (4 min)	1–3	3
Platelet factor 3	normal	normal
Platelet adhesion	normal	normal
Platelet aggregation 2 + 20 mm ADP, collagen and epinephrine	normal	normal

Case 2 L. M., a 12-year-old black male (BD 2 11 59) was well until 13 months of age when he began to have epistaxis, usually controllable by pressure. At 5 years of age, a 3 hour episode of epistaxis prompted investigation of the coagulation mechanism leading to the diagnosis of factor V deficiency. When the patient was 6 years old, an episode of gastrointestinal hemorrhage required transfusion. Radiographic studies of the upper and lower gastrointestinal failed to delineate the origin of the hemorrhage.

The child has done reasonably well with epistaxis controllable at home. He is extremely active and has sustained multiple lacerations and abrasions, all of which responded to local measures. A foot laceration required suturing but no replacement therapy. Bruises are constantly present, however, routine immunizations were not associated with hematomas. Eruption and shedding of the deciduous teeth have not been associated with hemorrhage.

At 11 years of age, he sustained a fracture of the left clavicle. The overlying hematoma measured 5×6 in, but no transfusions were necessary. The factor V level was found to be 2.5% (table I).

Family history revealed that the paternal grandmother was aware of easy bruising but had no hemorrhage following appendectomy, mastectomy, oophorectomy or delivery. There was no consanguinity. A sibling of the patient died in infancy of congenital hydrocephalus, two siblings are alive and well.

Coagulation Studies

Plastic syringes, pipettes and test tubes were utilized, except disposable glass serology tubes for the whole blood clotting time and clot retraction. Using a 2-syringe technique the blood was drawn with a 21 guage scalp vein needle, anticoagulated in the ratio of 9:1 with 3.8% citrate and placed on melting ice. The samples were centrifuged in a refrigerated centrifuge for 15 min at 10,000 rpm.

First stage factors XII, XI, IX, VIII were assayed using congenitally deficient plasmas in a modification of the PTT [16]. Reference plasma obtained from Hyland Laboratories or pooled normal plasma for factor XII were employed as standards. Commercial thromboplastin (Dade) and citrated normal plasma (Dade) were used for the prothrombin time control and to establish the standard curves on log-log paper for factor II, V, VII-X-II (PP) assays. Factor V substrate was prepared by the method of STEINBERG *et al.* [15]. Factor II deficient substrate was composed of equal parts of deprothrombinized beef plasma (Dade) and normal serum aged for 24 h at 37 °C. PP substrate was commercial deprothrombinized beef plasma (Dade).

All coagulation determinations were performed with a BBL Fibrometer.

Platelet Studies

Blood for platelet studies was similarly obtained but was not chilled. Platelet aggregation methods and materials were those of MUSTARD *et al.* [9]; platelet adhesion, the method of SALZMAN [11]. *Bleeding time was the Ivy technique.*

Platelet factor 3 (PF 3) was a modification of the method of HARDISTY and INGRAM [4] Briefly, the test is an incubation of platelet rich plasma (PRP) and platelet poor plasma (PPP) with kaolin for 20 min which is then calcified The absence of plasma factor V makes it impossible to determine if PF 3 was released by factor V deficient platelets in V deficient plasma because the clotting time is prolonged in the absence of factor V Therefore an additional 0 1 ml of normal or V deficient plasma was added immediately prior to calcification

Results

All parameters measured were normal except factor V levels, which was <1% in case 1 and 2 5% in case 2 (table I) Prothrombin times of PPP and PRP were similar

The platelets of our patients released PF 3 normally (table II) In the modification of PF-3 test it may be reasoned that the added factor V

Table I

	Case I	Case II
PTT (30–45 sec)	150	53–76
PT (10–11)	30 7 34 9	28 0–32 3
Clotting time (12 min)	20	17
Thrombin time (11–15 sec)	11 9	12 3
Fibrinogen (200–400 mg)	312	352
Factor assay %		
V	1	2 5
II	95–100	100
PP	75–88	96
VIII	114	150
IX	72	70
XI	109	74
XII	88	140
XIII	normal	normal
Inhibitor	negative	negative
Platelets × 10³	225 346	360 000
Clot retraction	good	good
Bleeding time (4 min)	1 3	3
Platelet factor 3	normal	normal
Platelet adhesion	normal	normal
Platelet aggregation 2+20 mm ADP collagen and epinephrine	normal	normal

Table II Platelet factor 3 release in factor V deficient patients

PRP	PPP	Added at 20 *minutes*	Clotting time, sec	
			case 1	case 2
C	C	C	30.5	33.4
P	C	C	31.0	33.4
P	P	P	117.0	87.5
P	P	C	32.0	34.5
P	C	P	31.3	32.0

C = Control normal plasma from RAS P = patient's plasma

allowed the factor V deficient platelets to release PF-3 after immediate absorption The time was probably insufficient because previously it had been demonstrated that factor V deficient platelets incubated for 10 min in deprothrombinized human plasma were less effective than a 10 fold dilution of normal platelets [7] Therefore, PF-3 was probably released normally by the factor V deficient platelets in factor V deficient plasma

The platelet aggregation responses to 2 and 20 μM ADP, epinephrine, and collagen were normal In case 1, these were the lower range of normal and were repeated 60 h after a plasma infusion when the factor V level was 4% No difference in the aggregation curves was detectable

Discussion

Plasma coagulation factors VIII and V adhere to the platelets [6] The binding forces for *in vitro* platelet aggregation appear to be independent of plasma factors V and VIII because normal platelet aggregation has previously been demonstrated in factor VIII deficiency [3] However, in hemophilia and a factor V deficient patient the primary bleeding time was normal, but when the crust was removed the secondary bleeding time was abnormally prolonged The infusion of normal platelets (in 35–65 ml of plasma) shortened the secondary bleeding time in factor V or factor VIII deficient patients for several days. It was concluded that survival of factors V and VIII on the platelet surface approached that of platelets rather than the half life of the plasma coagula

tion factors [1] Transfusion of fresh normal plasma produced a normal secondary bleeding time as long as the factor V level was 5–7% [2]

Of the 58 reported cases of factor V deficiency, bleeding times were recorded for 41 patients and were considered abnormal by the original author in 14 (34%) patients [13] The recently appreciated coagulation defect produced by aspirin makes it necessary to interpret these data with caution

Factor V deficiency appears to be a relatively mild hemorrhage diathesis Both our patients sustained lacerations large enough to require sutures without associated hemorrhage The major hemorrhagic manifestations in factor V deficiency are from the mucous membranes i e, epistaxis, hemorrhage with the eruption of shedding of the deciduous teeth and lacerations of the tongue, gums, or frenulum Excessive bruising is common but not troublesome The hemorrhagic episodes frequently respond to single infusions of fresh blood or plasma and deaths from hemorrhage are extremely rare [13]

EACA has been noted to be a helpful adjuvant in the management of patients with hemophilia and oral hemorrhage, as it inhibits normal fibrinolysis In our experience EACA has reduced the number of rebleeding episodes in hemophiliacs with oral lesions Thus, we have administered EACA routinely to all patients with hemorrhagic diathesis and oral lesions

Hemorrhage during the neonatal period appears to be distinctly unusual One infant with a labial hematoma was diagnosed in the perinatal period [12] Umbilical hemorrhage was recorded in another case, who was diagnosed later in life [5] The only other notation, a fatal umbilical hemorrhage, was in an untested sibling of a patient with factor V deficiency [10]

Although only 58 cases of factor V deficiency have been recorded following the original description in 1947, the disease may not be as rare as previously thought. The finding of 2 unrelated cases reflects an increased awareness of hemorrhagic disorders and readily available coagulation studies

Factor V deficiency is usually considered to be inherited as an autosomal recessive condition [13] The mother and maternal brother and sister of case 1 have somewhat low factor V levels (66, 66, 55%), however, the maternal grandparents were both over 100% The parents and sibling of the second patient have normal factor V levels (85, 74, 89%) At present no definite statement appears possible regarding the detection of the heterozygote

Case Report

V. R., a 70-year old farmer, came under our observation on June 25, 1968. The family history was negative. In 1960 he began to suffer chronic bronchitis. In January 1968 remittent fever with diffuse thoracic pain was established and lasted until April of the same year. From then on the patient suffered sporadic febrile episodes, edema at the lower extremities, weakness. He never showed purpuric manifestations. The physical examination revealed hyponutrition and pallor. He presented with slight malleolar edemas. There was no significant lymphadenopathy. A slight hepatomegaly and a discrete splenomegaly were present. There was a pleural effusion on the right hemithorax. The cardiac area was slightly enlarged towards the left. Pulse rate was 80/min and the blood pressure 110/70 mm Hg.

The patient's blood, soon after the drawing, underwent a massive gelation at room temperature. This did not occur when the blood was drawn with a warm syringe and immediately placed in a thermostat at 37 °C. Even slight decrease of temperature caused irreversible gelation.

Main laboratory findings: Urine: specific gravity 1.020–1.027, daily diuresis about 1,500 ml, urea 20 g/day, slight albuminuria, the microscopic examination of the urinary sediment was normal. Blood urea nitrogen 70 mg%. Red blood cells 1.9 million/mm³. Hemoglobin 6.1 g%. White blood cells 9,000/mm³. Differential count: 35% polymorphonuclear leukocytes, 57% lymphocytes and 8% monocytes. Platelets 230,000/mm³. Reticulocytes 1%. Erytrocyte sedimentation rate 120 mm after 1 h (Westergren method). Ra latex fixation test and Sia water test negative.

The radiological examination revealed a shadow on the left pulmonary base, widening of the superior mediastinum, especially on the right side. The pleural liquid extracted by thoracentesis presented the characteristics of a transudate (absence of cryoglobulins). The sternal myelobiopsy was characterized by a definite hyperplasia of lymphoreticular elements (30% of the myelogram) with parenchymal hypoplasia and shift to the left of the erythroblastic maturation curve. The liver needle biopsy showed infiltration of the portal spaces by lymphocytes. A slight regressive phenomenon was shown in the parenchyma. The capillaroscopy of the nail bed and of the bulbar conjunctive showed a rather conspicuous irregularity in the caliber and in the direction of the capillary loops, with evidence of the sludge phenomenon.

Blood protein was 7.26 g%, whereas blood protein obtained from the supernatant after the removal of cryogel was 6.36 g%. The electrophoresis on cellulose acetate at 37 °C, after the dissolving of the cryogel with urea, and at room temperature after separation of the cryogel, showed in all strips an evident band separation defect. But the reduction of the β_2-peak in the serum filtrate was clearly evident (fig. 1). The gel was completely dissolved by a 2 M solution of urea and it was reformed when urea was removed by dialysis; however, it redissolved when the last dialysis was done. The gelation was inhibited because of the acid medium; the gel did not form at lower pH values than 6 (fig. 1). No effect was seen by concentrated saline solutions (NaCl from 2 to 5 M).

On urea solubilized cryogel the separation was performed on discontinuous sucrose gradient containing 2 M urea (1.2 ml of sucrose at 40%, 1.2 at 30%, 1.2 at 20%). The 10-percent sucrose layer was not used because it became permeable by the cry

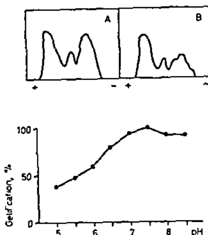


Fig 1 Top electrophoretic patterns of serum proteins on cellulose acetate, in A at 37 °C in B at room temperature after cryogel separation Bottom cryogelification of the serum placed at 37 °C at scalar pH values

oprotein For each tube, 15 OD in 1 ml of cryoprotein was allowed to settle The preparative ultracentrifugation was done with Spinco apparatus L2 65, with rotator SW-65, in tubes of 5 ml at 50 000 rpm (179 000 *g*) at 5 °C In 5 hours of centrifugation, 2 peaks were separated (fig 2) a heavy one equal to 82 6% (sedimentation coefficient 21 22S) and a light one equal to 17 4% (s c 7S)

The simple radial immunodiffusion test on the gradient separated fractions showed that the heavy and the light peaks consisted specifically of immunoglobulins with IgG and IgM specifity (fig 3) The immunoelectrophoresis showed the presence of a thick IgM band and a thin IgG band in the heavy peak A thick IgG band and a thin IgM band were present in the light peak (fig 3) The Ra latex test was negative in both fractions

The 21 22S fraction was labelled with ^{125}I (specific activity 115 μc/mg) This fraction in the autoradiography of the electrophoretic strip formed a single radioactive band at the initial point of migration (fig 4, at top) A dose of 100 μc was then injected into the patient Samples of heparinized blood (6 ml) were then drawn after 10 min and after 1 h for calculation of plasma volume The same samples were taken once a day for 22 days The samples were immediately placed at 37 °C, plasma after gelation at room temperature was dissolved in a solution of 2 M urea for counting in a well scintillator The radioactivity was also inspected for urinary proteins Figure 4 shows the plasma radioactivity disappearance curve after the injection of the heavy ^{125}I labelled cryogel ultracentrifugal fraction The curve consists of 2 exponentials and is characterized by a step rise as regards the normal γ globulins Below are reported the turnover values obtained by the method of MATHEWS [16]

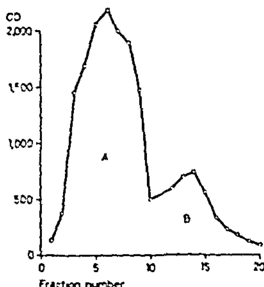


Fig. 2. Ultracentrifugal pattern of the serum cryogel dissolved in 2 M urea (ultracentrifugation at 50,000 rpm for 5 min). The first peak, equal to 82.6%, has a 21-22S sedimentation constant, the second, equal to 17.4%, has a 7S sedimentation constant.

which is based on the exponential calculation of the b_1 and b_2 and the a_1 and a_2 intersections of the components of the curve.

Fractional catabolic rate = 53.2% per day, plasma volume = 3 375 ml, cryoglobulinemia = 2.17 g%, plasmatic compartment = 71.5 g, absolute catabolic rate = 37.8 g/day, extravascular compartment = 160 g, total pool = 231.5 g.

The patient left the hospital 2 months after the date of admission in a serious cachectic state. He did not show substantial modification of the laboratory findings during his hospital stay, and died a month later.

Comment

Characteristic of the patient's blood behavior was gelation, at temperature slightly lower than 37 °C, of a cryoprotein complex formed by γM and γG globulins. The disease must be placed among the immunoproliferative ones, affecting particularly the γM-class of immunoglobulin-forming cells. This fact is confirmed by the presence of a macroglobulinic component with IgM specifity in the cryoprecipitate. The hematological peripheral and medullary findings, together with the liver biopsy, also contribute to this hypothesis by showing a clear hyperplasia of the lymphatic component, as in Waldenström's disease. But, because of the high blood

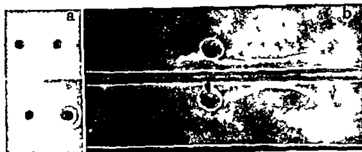


Fig 3 S mple d ffus on test (a) and immunoelectrophores s (b) of the heavy and the light peak separated by ultracentr fugat on The s mple d ffus on test shows that the 21 22S component (on the r ght) and the 7S component (on the left) have an IgG IgM specif ty (ant IgG serum above and anti IgM below) The immunoelec trophores s of the heavy (above) and l ght peak (below) aga nst horse ant human globul n serum (ant IgG IgM IgA) shows that the heavy peak predom nantly cons sts of IgM with a narrow IgG arc Also in the 7S fract on an IgM compo nent s present

cryoglobulin content and in particular the composition of the cryopre cipitate th s form may be comparable to the type of essential cryoglobuli nemia based on the interaction between a 19S paraprotein and a 7S glob ulin [8]

The sucrose gradient separation gave 2 peaks with prevalence of the heavy peak The typing showed that both peaks consisted of globulins with γM and γG specifity The heavy peak was nearly represented by ma croglobulin with a small quantity of 7S globulins with IgG specifity bound to the macroglobulin molecules in order to form the 21 22S complexes The sedimentation constant was slightly lower than the values found for the 7S 19S complexes (e g •rheumatoid factor 7S) In addition the bond between macrogloublin and 7S was remarkably stable This was not the case with 7S 19S complexes and with the 7S 19S cryo precipitating-complexes of the double cryoglobulinemia with reversible precipitation Therefore the sedimentation coefficient of the heavy peak slightly lower than that shown by the 19 7S complexes could be due to the presence in the peak of unbound macroglobulins together with the ones shown This behavior could also be attributed to a different molecu lar weight of the macroglobulin with sedimentation coefficient lower than 19S as frequently found in patients with Waldenstrom s disease The 7S peak in the immunodiffusion test and in the immunoelectrophoresis also

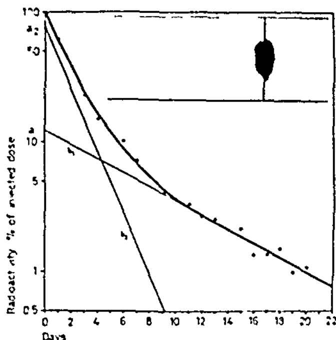


Fig. 4. Plasma radioactivity disappearance curve after injection of 21-22S fraction labelled with ¹³¹I. Top: autoradiography of cellulose acetate electrophoretic strip of the same fraction obtained by ultracentrifugation.

appeared to consist of a protein with IgG and IgM specifity. This unexpected behavior was surely due to the presence in the peak of macroglobulin degradation products, probably of 6.5-7S monomers. The negativity of the latex test for rheumatoid factor in the serum at 37 °C and in the cryogel suggests that the reactivity of the cryogel macroglobulin for the IgG may be peculiar and not shown by passive agglutination tests.

The presence of 7S-19S macromolecular complexes in the circulating blood explains the capillary alteration in the nail bed and in the bulbar conjunctive, even in absence of macroscopic alterations of the distal cutaneous territories.

The notable difference of the 19S-7S bond in the present case with respect to the rheumatoid factor-7S bond and to the macrocryoglobulin-7S bond (which is found in the more frequent form of double cryoglobulinemia) should be underlined. Probably this bond belongs to the antigen-antibody type. This should be confirmed by the study of FRANKLIN [17] who found that the bond which determines the cryogelation is stable and not covalent.

The metabolism of heavier aggregates is very accelerated, going beyond the turnover values of the normal and pathological globulins This fact may be due above all to the degree of complex denaturation, very likely connected with the particular type of bond between the two immunoglobulins A previous denaturation *in vitro* can be excluded because the electrophoresis on cellulose acetate of the labelled complex showed a single narrow band, and the turnover study was done immediately after labelling Also, the prevalence of the extravascular compartment on the plasmatic complex, unusual for the normal macroglobulins [18, 19] and, to a lesser degree, for the pathological macroglobulins [19–22], must be attributed to the elevated degree of complex denaturation, which conditions the conspicuous intercompartmental exchange of 7S-19S fragments This finding, moreover, is documented by the presence of fragments with IgM specifity in the gradient's 7S peak.

The paraprotein hypercatabolism could explain the hyperazotemic state of the patient, even if a failure of the renal function was not clinically evident The abnormal level of synthesis and catabolism of paraprotein (37 8 g/day) could partly justify the serious state of the patient, even if no organ lesion could be shown (with the exception of the anemia, due to the medullary hypoplasia, secondary to the lymphoreticular metaplasia) The course of the disease was particularly short (8 months), considering the average survival of patients affected by noncomplicated paraproteinemias

References

1 Lerner A B and Watson, C. J.. Studies of cryoglobulins I Unusual purpura associated with the presence of a high concentration of cryoglobulin (cold precipitable serum globulin) Amer J med Sci *214* 410 (1947)

2 Barr, D P, Reader, G G., and Wheeler, C. H Cryoglobulinemia I Report of 2 cases with discussion of clinical manifestations incidence and significance Ann intern Med *32* 6 (1950)

3 Ritzmann, S E. and Levin, W C. Cryopathies a review Classification, diagnostic and therapeutic considerations Arch intern Med. *107* 754 (1961)

4 Caronia, F Guarnaccia C, Chiappara, A e Mauro B Su di un caso di crioglobulinemia essenziale Studio clinico e sierologico Haemat. lat., Milano 9 201 (1966)

5 Bonsdorff, B Von, Groth H., and Packalen, T.. On the presence of a high molecular crystallizable protein in blood serum in myeloma. Folia haemat., N F 59 184 (1938)

6 VOLPÉ, H R, BRUCE ROBERTSON A, FLETCHER A A, and CHARLES W B Essential cryoglobulinemia Review of literature and report of case treated with ACTH and cortisone Amer J Med 20 533 (1956)
7 POWERS J H Essential cryoglobulinemia manifested by peripheral gangrena and massive purpura report of a case Amer J clin Path 30 58 (1958)
8 LOSPALLUTO J DORWARD, B, MILLER, W, jr, and ZIFF, M Cryoglobulinemia based on interaction between a gamma macroglobulin and 7S gamma globulin Amer J Med 32 142 (1962)
9 CARONIA, F, PATTI A, GUARNACCIA, C e MAURO B Caratteristiche immunologiche e metaboliche di una macrocrioglobulina attiva come fattore reumatoide Minerva med 60 2054 (1969)
10 SHUBOTT, H Serologische und klinische Befunde bei Patienten mit Kryogelproteinamie Schweiz med Wschr 88 1035 (1958)
11 IMHOF, J W, BAARS H, and VERLOOP, M C Proc 6th Congr Europ Soc Haemat, Copenhagen 1957
12 WESTENDORP BOERMA F and MANDEMA, E A serologic investigation of Waldenstrom's macroglobulinemia by means of the agar diffusion method J Lab clin Med 49 358 (1957)
13 BRAUNSTEINER H FALKNER, R NEUMAYER, A und PAKESCH F Makromolekulare Kryoglobulinaemia Klin Wschr 32 722 (1954)
14 RITZMANN, S E, THURM R H, TRUAX, W E, and LEVIN, W C The syndrome of macroglobulinemia review of literature and report of 2 cases of macrocryogelglobulinemia Arch intern Med 1055 939 (1960)
15 NUTTER D O and KRAMER N C Macrocryogelglobulinemia report of a case with unusual spontaneous recovery Amer J Med 38 462 (1965)
16 MATTHEWS C M E The theory of tracer experiments with 131I labelled plasma proteins Phys Med Biol 2 36 (1957)
17 FRANKLIN E C The immune globulins Their structure and function and some techniques for their isolation Progr Allergy, vol 8 p 58 (Karger Basel 1964)
18 COHEN S and FREEMAN T Metabolic heterogeneity of human gamma globulin Biochem J 76 475 (1960)
19 BARTH W F, WOCHNER R D, WALDMANN T A, and FAHEY J L Metabolism of human gamma macroglobulins J clin Invest 43 1036 (1964)
20 GABUZDA T G The turnover and distribution of 131I labelled myeloma and macroglobulin proteins J Lab clin Med 59 65 (1962)
21 OLESEN H Turnover studies with iodine labelled gamma macroglobulin and albumin Scand J clin Lab Invest 15 497 (1963)
22 WILKINSON P DAVIDSON W and SCHMARTZ A Turnover of 131I labelled autologous macroglobulin in Waldenström's macroglobulinemia Ann intern Med 65 308 (1966)

Authors address Prof F CARONIA Dr A PATTI Dr G DONATUTI and Dr C GUARNACCIA Istituto di Patologia Medica Policlinico Feliciuzza I 90133 Palermo (Italy)

H. HUBER, D. PASTNER und F. GABL (mit Vorwort von H. BRAUNSTEINER) unter Mitarbeit von H. ASAMER, W. R. MAYR und F. SCHMALZL. Laboratoriumsdiagnose hämatologischer und immunologischer Erkrankungen. Springer, Berlin 1972. XXIV + 381 pp., 36 fig., US $ 21.20 / DM 68.–

Laboratoriumsuntersuchungen sind aus der modernen Diagnostik nicht mehr wegzudenken, ja, die Möglichkeiten des Laboratoriums werden oft als unbegrenzt angesehen. Dabei stellen sich dem Arzt immer wieder zwei Fragen: Was für Laboratoriumsuntersuchungen sind bei einem bestimmten Fall aus der Vielzahl von Testen am zweckmässigsten auszuwählen und wie soll ein bestimmtes Laboratoriumsresultat interpretiert oder in die klinische Diagnostik integriert werden? Das aus der Innsbrucker Schule hervorgegangene Buch gibt Antwort auf diese beiden Fragen. Die Verbindung eines methodischen Teils mit einem allgemein diagnostischen Teil hebt das Werk hoch über die üblichen Laboratoriums-«Kochbücher» heraus. Der diagnostische Teil erstreckt sich von Diagnose und Differentialdiagnose hämolytischer Anämien bis zu den serologischen Methoden zur Spenderauswahl bei Organtransplantationen. In jedem Kapitel werden die einzelnen zur Verfügung stehenden Tests kurz besprochen und gewertet. Sodann wird der Untersuchungsgang angegeben. Es findet sich jeweils auch eine kurze Beschreibung der einzelnen Krankheitsbilder.

Der zweite methodische Teil bringt eine genaue Beschreibung der einzelnen Methoden, wobei in einer kurzen Besprechung jeweils die Bedeutung des Tests, besondere Schwierigkeiten usw. unterstrichen werden. Die Gliederung der Methoden entspricht im wesentlichen derjenigen des ersten diagnostischen Teils. Der methodische Teil ist sehr sorgfältig ausgearbeitet. Man spürt auf Schritt und Tritt, dass den beschriebenen Methoden eine grosse persönliche Erfahrung zugrunde liegt. Diese persönliche Note, welche der Referent als besonders wertvoll hervorheben möchte, führt notgedrungen zu gewissen Einseitigkeiten. Der Schwerpunkt des Interessengebietes der Autoren liegt zweifellos bei der Immunologie. Der Anwendungsbereich immunologischer Methoden wird dementsprechend umfassend dargestellt. Es wird deutlich, dass die Immunologie nicht nur bei der Abklärung der eigentlichen Krankheiten des Immunsystems und in der Hämatologie unentbehrlich ist, sondern auch in vielen andern Gebieten wertvolle Dienste leistet. Dies geht besonders aus den Kapiteln über den Lupus erythematodes, den Rheumafaktor und die immunologischen Untersuchungen bei Leber- und Nierenkrankheiten hervor. Die Auswahl der beschriebenen serologischen und immunologischen Methoden ist sehr geschickt getroffen worden. Als sinnvolle Ergänzung fügt sich ein Kapitel über Immunfluoreszenz [ASAMER] und über Gewebstypisierung [MAYR] ein. Aber auch die zytochemischen Methoden erfahren eine ihrer Bedeutung entsprechende Darstellung [SCHMALZL]. Das gleiche gilt von den übrigen der Abklärung von Anämien dienenden Methoden. Es scheint verständlich, dass auf eine Darstellung der hämatologischen Grundmethoden (Zellzählung, Hämoglobinbestimmung usw.) verzichtet wird. Weniger gut einzusehen ist dagegen, warum gewisse Isotopenmethoden aufgenommen sind (Schillingtest, Vitamin-B_{12}-Bestimmung), andere dagegen nicht. Auch fehlt eine Darstellung der Methoden zur Folsäurebestimmung oder zum Porphyrinnachweis. Ferner wäre es vielleicht nützlich, wenn Alternativen des

LE-Zellennachweises angegeben wurden. Auf den Einschluss von Gerinnungsuntersuchungen wurde absichtlich verzichtet.

Gesamthaft bieten sowohl der allgemeine wie der methodische Teil einen hervorragenden Überblick über die moderne Diagnostik hämatologischer Erkrankungen. (Als Anregung für die sicher bald notwendige zweite Auflage möchte der Referent um den Verzicht auf allzu viele Abkürzungen bitten.) Das Buch gehört nicht nur in jedes Spitallaboratorium, sondern bietet auch dem praktisch tätigen Internisten viele Informationsmöglichkeiten. U. BUCHER, *Bern*

Varia

International Committee for Standardization in Hematology

Availability of Hemiglobincyanide Reference Preparation

An international hemiglobincyanide reference solution is prepared by the Rijks Instituut voor de Volksgezondheid, Bilthoven, the Netherlands, under the auspices of the International Committee for Standardization in Hematology (ICSH) [Brit. J. Haemat. *13*, suppl. 71, 1967]. The project is sponsered by the Council of Europe and by the World Health Organization. The latter authority has established the solution as International Hemiglobincyanide Reference Preparation [Wld Hlth Org. techn. Ser. *384*, 12, 86, 1968]. It is available free of charge on request to national laboratories working in hematology or to interested individual workers, and is intended for checking the purity and content of hemiglobincyanide reference solutions to be used in the daily practice of hemoglobinometry. It consists of an aqueous hemiglobincyanide solution equivalent to a hemoglobin content of approximately 60 mg/100 ml, dispensed in 10 ml ampoules and packed in boxes of six. It is tested and regularly checked by a number of control laboratories nominated by ICSH.

Further information may be obtained from Dr. A. H. HOLTZ, Secretary ICSH Expert Panel on Hemoglobinometry, Rijks Instituut voor de Volksgezondheid, P.O. Box 1, *Bilthoven* (The Netherlands).

Third Mediterranean Congress on Thromboembolism

The Third Mediterranean Congress on Thromboembolism will take place during the first week of June 1973 in Tel Aviv.

Main topics: 1. Pathogenesis: (a) vascular factors, (b) platelets, (c) coagulation factors. 2. Diagnostic procedures. 3. Clinical aspects. 4. Therapeutic aspects: (a) fibrinolytic, (b) anticoagulants, (c) surgical.

The language of the Congress is English.

President: Dr. A. DE VRIES; Secretary: Dr. I. COHEN.

Additional informations can be obtained from the Organizing Committee, the Third Mediterranean Congress on Thromboembolism, P.O.B. 16271, *Tel Aviv* (Israel).

Acta Haemat. 48 193-201 (1972)

Enzymatic Activities and Glutathione Content of Erythrocytes in the Newborn: Comparison with Red Cells of Older Normal Subjects and those with Comparable Reticulocytosis[1]

PATRICIA N KONRAD, W N VALENTINE and D E PAGLIA

Department of Pediatrics Medicine and Pathology UCLA Center for the Health Sciences, and Wadsworth General Hospital, Veterans Administrations Center, Los Angeles, Calif

Abstract 20 enzymatic activities and the glutathione content of newborn erythrocytes are compared (a) to normal and (b) to com parably reticulocyte rich nonneonatal red cells Six were very high in comparison to either control group (GSH PGK, Enol., G 3 PD GPI, G-6-PD) Five were consistently very low (ACHE, RPK, GSH Px, AK and PFK) The mean of the remainder differed from the mean of comparably reticulocyte-rich blood by less than 1 SD of the latter mean. Cord erythrocytes exhibit a characteristic metabolic pattern not explained by a young mean cell age alone

Key Words
Erythrocytes
Newborn
Enzymes
Glutathione

While a number of features of erythrocyte metabolism in the newborn infant have been previously investigated, comparison has frequently been made with the red cells of the normal adult only Moreover, the range of activities measured has usually been limited in any one study, and a variety of methodologies have been employed [22] Red cells of newborn infants glycolize more actively [16, 21], and possess higher levels of ATP and adenine nucleotides than do adult erythrocytes of normal mean cell age [8, 12, 21, 28] However, when comparison has been made with appropriately young adult red cell populations from subjects with reticulocytosis, the young cells of the neonate appear to consume less glucose than expected [24] The activities of a number of enzymes of the Embden-Myerhof and pentose phosphate shunt pathway have been shown to be

[1] Supported by research grant HL 12944 from the National Institutes of Health and Leukemia Research Foundation, Los Angeles, Calif

greater than (or at least equal to) those of normal adult red cells (Hexokinase, aldolase, glyceraldehyde-3 phosphate dehydrogenase, phosphoglycerate kinase, enolase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate and 6-phosphogluconate dehydrogenases) [5–7, 9, 11, 12, 23, 28–30, 39–41]. Red cell glutathione may also be increased above that in the adult [31, 41]. In contrast, and of considerable interest, is the observed decrease in the activities of phosphofructokinase [5, 12, 23, 39], glutathione peroxidase [10, 20, 38] and acetylcholinesterase [4, 14] in erythrocytes of newborn infants. It is believed that the mean cell age of cord erythrocytes is younger than that of normal adult blood, and, indeed, the low grade reticulocytosis of the newborn is a routine finding [22]. Young erythrocytes characteristically possess higher activities of many enzymes than those of older cells [1, 17, 26].

Presumably each enzyme protein, incapable of being replenished in the mature erythrocyte, has its own biologic half life. The very stable moieties show little change in activity as the cell ages, the more labile proteins decay at varying rates. The differences in enzymatic profile of cord and nonneonatal erythrocytes have only rarely been defined in terms of which features are attributable to mean cell age and which are truly unique for the red cells of the newborn. WITT *et al.* [39], on the basis of density gradient separation of blood of newborn and adults into younger and older fractions, have concluded that increased activities of pyruvate kinase and glucose-6-phosphate dehydrogenase are in the case of the former completely, and in the case of the latter partially, dependent upon the reticulocyte content of cord blood. OSKI [23], in studies directly comparing cord erythrocytes with reticulocyte-rich blood from adults, has emphasized that the degree of increase in enolase and phosphoglycerate kinase activities in red cells of the newborn are unique and not explainable by reticulocyte content alone. The same investigator has emphasized that deficiency of phosphofructokinase in cord blood is accentuated when comparison to reticulocyte rich adult blood is appropriately made. The present study compares metabolic activities of cord erythrocytes with those of both normal, nonneonatal blood and nonneonatal blood with comparable reticulocytosis.

Materials and Methods

Metabolic Studies

The following enzymatic activities of saline washed erythrocytes harvested by differential sedimentation were assayed by previously reported methods [2, 13, 15

18, 25, 27, 32, 33, 35–37] hexokinase (HK) glucosephosphate isomerase (GPI), phosphofructokinase (PFK) fructosediphosphate aldolase (Ald), triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (G 3-PD), phosphoglycerate kinase (PGK), phosphoglyceromutase (PGM), phosphopyruvate hydratase (enolase-Enol), pyruvate kinase (PK) lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G-6-PD) 6-phosphogluconate dehydrogenase (6-PGD), glutathione peroxidase (GSH Px), lactoyl glutathione lyase (Glyoxalase I) hydroxy acyl glutathione hydrolase (Glyoxalase II), adenylate kinase (AK) ribosephosphate pyrophosphokinase (RPK, PRPP synthetase) and acetylcholinesterase (AChE) The distal pentose shunt (DPS) was screened in terms of production of fructose-6-phosphate (F-6-P) from ribose 5-phosphate (R 5-P) The enzymes involved in the reaction sequence are ribulosephosphate-3-epimerase phosphoriboisomerase, transketolase and transaldolase PK was assayed as previously described except that substrate phosphoenolpyruvate (PEP) was increased to 3 mM and adenosine diphosphate (ADP) to 2 mM final concentration. Red cell glutathione (GSH) was measured according to BEUTLER *et al* [3] employing 5,5-dithiobis-(2 nitrobenzoic acid) All data are expressed in terms of 10^{10} erythrocytes.

Clinical Material

The above activities were compared in three groups of subjects

Group A This group consisted of subjects serving as normal controls for investigations conducted on patients with hemolytic syndromes in our laboratory All were deemed clinically well, and none had anemia or reticulocytosis The means and standard deviations for 13 of the measurements were derived from 42 to 52 individual controls The data for 7 (LDH, GSH Px, AK, Glyoxalases I and II, RPK, and GSH) were obtained from assays on 22–27 subjects Due to a comparatively recent change in the substrate concentrations employed in routine assay, information relative to PK was obtained in only 12 subjects in group A and 8 subjects in group B

Group B This group comprised nonneonatal subjects studied in our laboratory over the past 6 years because of uncomplicated hemolytic syndromes and reticulocytosis ranging from 3 0 to 8 9% The group was selected to provide cell populations whose reticulocyte counts approximated those of cord bloods While the etiologies of the chronic hemolytic syndromes were varied, most were hereditary It is a heterogeneous group in terms of age with both adults and children included It has been the experience of this laboratory over many years that red cell enzyme activities in [illegible]

[illegible]

therapy in the latter, enzymatic activities are the mean resultant of a

new, young population, and older cells circulating prior to therapy. Such bimodal populations were deemed unsatisfactory for inclusion in the reticulocyte-rich control group. For 14 measurements, data were derived from 21 to 43 subjects. Five (LDH, GSH-Px, Glyoxalase I and II and GSH) were measured in 13–18 subjects. Hereditary deficiency states were, of course, excluded from data relating to the deficient enzyme.

Group C. Cord blood samples obtained from 22 normal, full-term infants were studied and the results compared with groups A and B. Birth weights ranged from 2,560 to 4,200 g with all but four being between 3,000 and 4,000 g. The packed cell volume (PCV) of samples studied varied from 39 to 56 ml/100 ml of blood with a mean of 48.2 ± 4.5. Reticulocyte percentages ranged from 2.3 to 7.0 with a mean of 3.8 ± 1.0. 14 infants were male and 8 female.

Results and Conclusions

Results are summarized in tables I, II and III. In order to achieve as meaningful a comparison as possible, special emphasis has been placed on the relationships between cord blood and group B, since reticulocytosis of roughly similar degree was present in both. The tabular data make it immediately apparent that quite different conclusions may be drawn if the factor of reticulocytosis and mean cell age is neglected. In table I, therefore, a given metabolic activity is arbitrarily designated as high if its mean exceeds the mean for the same measurements for group B cells by 1 or more SD of the group B mean. The six values meeting this criteria are arranged in order, with the highest listed first. Table II records mean values which lie within 1 SD of the mean of group B. In table III, low values are defined as those more than 1 SD below the group B mean with the exception of those for ACHE and RPK where adequate comparative data are available only with group A.

It is clear that cord erythrocytes have a characteristic pattern of enzymatic activities. Also GSH, which is normally increased little, if at all, in reticulocyte-rich blood, is consistently elevated in cord blood. Five of the six values shown in table I are more than 2, and three are more than 3 SD above those of group B erythrocytes. The uniquely elevated activities of PGK and enolase in neonate cells reported by others [23, 39] are confirmed, but those of G-3-PD and GPI are also markedly increased. In fact, with the exception of G-6-PD, all values noted in table I have been, in our experience, far above those we have encountered in a large group

Table I Metabolism of cord blood (group C) erythrocytes values higher than nonneonatal blood

Parameter	Assay units[1]			Group C compared[2] to	
	group A	group B	group C	group A	group B
GSH	666.0 ± 118	655.0 ± 103	1041.0 ± 134	+3.2	+3.7
PGK	25.5 ± 3.7	27.7 ± 4.6	42.0 ± 2.8	+4.5	+3.1
Enol	3.8 ± 0.9	5.3 ± 1.4	9.5 ± 1.1	+6.3	+3.0
G 3 PD	30.3 ± 4.5	35.5 ± 6.8	51.6 ± 5.5	+4.7	+2.4
GPI	12.7 ± 1.8	14.6 ± 2.5	20.6 ± 2.2	+4.4	+2.4
G-6-PD	2.7 ± 0.6	3.6 ± 1.0	4.7 ± 0.9	+3.3	+1.1

[1] Enzymatic activities are in arbitrary units as described in reference publications cited under Methods. GSH is expressed as $\mu g/10^{10}$ red cells. Mean and standard deviations are given.

[2] The difference between the *mean* of group C (cord erythrocytes) and *mean* of groups A and B was divided by the standard deviations of means of groups A and B, respectively. The number of standard deviations characterizing the difference in means in each instance is denoted + if the means of group C is the higher and − if it is the lower.

Table II Metabolism of cord blood (group C) erythrocytes values similar to nonneonatal blood

Parameter	Assay units[1]			Group C compared[2] to	
	group A	group B	group C	group A	group B
LDH	44.9 ± 6.1	53.3 ± 10.0	59.4 ± 7.8	+2.4	+0.6
Glyoxalase I	49.4 ± 10.0	60.2 ± 11.0	63.2 ± 6.6	+1.4	+0.3
HK	0.23 ± 0.07	0.51 ± 0.2	0.55 ± 0.12	+4.6	+0.2
6-PG D	1.8 ± 0.3	2.6 ± 0.6	2.7 ± 0.4	+3.0	+0.2
Ald	1.0 ± 0.2	1.5 ± 0.4	1.4 ± 0.3	+2.0	−0.3
DPS	12.7 ± 2.8	19.1 ± 4.3	17.3 ± 4.3	+1.6	−0.4
PK	4.5 ± 0.7	7.7 ± 1.4	7.2 ± 1.2	+4.4	−0.4
Glyoxalase II	16.8 ± 2.1	19.8 ± 3.3	17.5 ± 2.7	+0.3	−0.7
TPI	163.0 ± 21.5	184.0 ± 25.0	165.0 ± 12.0	+0.1	−0.8
PGM	7.8 ± 1.5	10.5 ± 2.8	9.5 ± 1.1	+1.2	−0.4

[1] Arbitrary units as described in reference publications cited under Methods.

[2] Basis of comparison same as for table I

Table III Metabolism of cord blood (group C) erythrocytes values lower than nonneonatal blood

Parameter	Assay units[1]			Group C compared[2] to	
	group A	group B	group C	group A	group B
ACHE	100 0 ± 9 0	– –	59 2 ± 8 3	–4 5	–
RPK	30 5 ± 3 8	– –	21 9 ± 4 7	–2 3	–
GSH Px	7 2 ± 1 8	9 6 ± 2 0	4 0 ± 1 1	–1 8	–2 8
AK	77 8 ± 16 8	85 1 ± 17 5	61 2 ± 8 3	–1 0	–1 4
PFK	3 0 ± 0 6	4 1 ± 1 1	2 9 ± 0 4	–0 2	–1 1

[1] Arbitrary units as described in reference publications cited under Methods ACHE expressed in percent normal mean for the laboratory Insufficient data exist on group B for comparison of ACHE and RPK (PRPP synthetase) Both enzymes characteristically have increased activities in young erythrocytes

[2] Basis of comparison same as for table I

of subjects with hemolytic syndromes, much younger cell populations, and high grade reticulocytoses of 10–22%.

Table II indicates that certain activities are not widely disparate in value from those found in erythrocytes of group B The necessity of making comparisons with group B as well as group A is strikingly emphasized here HK and PK, for example, are far more active in cord blood than in nonneonatal red cells of normal mean cell age Comparison with group B, however, indicates that these differences are no more than expected for the degree of reticulocytosis present They reflect a characteristic of young red cells rather than of cord erythrocytes *per se*

Table III confirms that certain activities are well below those expected for nonneonatal cells The low activities of ACHE, GSH Px and PFK confirm previous reports, although in our series low PFK was most effectively demonstrated in comparison with group B blood AK, an enzyme whose activity varies only modestly between old and young erythrocytes of nonneonatal blood exhibited consistently low activity in cord cells It should be emphasized that while adequate comparative data are available only with group A in the case of ACHE and RPK, these activities are clearly increased in young cells Comparison with comparably reticulocyte rich blood would accentuate further the obviously low activities in cord erythrocytes

Discussion

There is a highly consistent pattern to the metabolism of cord erythrocytes Such a pattern conceivably could represent persistence of a unique fetal erythropoiesis [22], doomed to disappear soon after birth in a manner analogous to fetal hemoglobin On the other hand, the pattern observed could result from the repression or depression of the synthesis of specific enzymes by metabolites peculiar to the metabolic milieu of intrauterine development In any event, the strikingly high and low values of tables I and III are not explainable in terms of either young mean cell age or the moderate macrocytosis of newborn cells The pattern differs vastly from that seen in pure hemolytic syndromes beyond the neonatal period of life It resembles in some, but not in all, respects abnormalities we have noted in folate and B_{12} deficiencies [19], and in certain hereditary and acquired syndromes characterized by ineffective erythropoiesis and karyo and cytokinetic abnormalities [34]

Acknowledgements The authors gratefully acknowledge the technical assistance of Mr Ernesto Guerque, Mr Klaus Kürschner, Mrs. Annaliese Merlino and Mrs. Julia Wittenberg

References

1 Allison A C. and Burn, G P Enzyme activity as a function of age in the hu

2 [illegible] Blood 32 236–249 (1968)

3 Beutler, E, Duron, O., and Kelly, B M Improved method for the determination of blood glutathione J Lab clin Med. *61* 882–888 (1963)

4 Burman D Red cell cholinesterase in infancy and childhood Arch Dis. Childh *36* 363–365 (1961)

5 Caruso P, Conti, F e Londrillo, A Diagramma della attività enzimatiche endoeritrocitarie nel neonato nel lattante, nel bambino Minerva pediat *15* 1136–1146 (1963)

6 Caruso, P Ricca M e Conti, F. Corredo enzimatico nell immaturo Minerva pediat. *15* 1146–1149 (1963)

7 Caveon, B von, Weber, H und Stamm O Enzymstatus bei normalen Neugeborenen, Frühgeburten, connatalen Erkrankungen und mütterlichen Schwangerschaftserkrankungen Gynaecologia *162* 341–349 (1966)

8 DeLuca, C., Stevenson, J H, Jr., and Kaplan E Simultaneous multiple-column chromatography its application to the separation of the adenine nucleotides of human erythrocytes Analyt Biochem *4* 39–45 (1962)

9 Fois, A., Biagoli, M L e Contu, L. Comportamento della glucosio-6-fosfato

deidrogenasi e del glutatione ridotto negli eritrociti dei neonati a termine e dei prematuri. Haematologica *46*: 178–196 (1961).

10 GROSS, R. T.; BRACCI, R.; RUDOLPH, N.; SCHROEDER, E., and KOCHEN, J. A.: Hydrogen peroxide toxicity and detoxification in erythrocytes of newborn infants. Blood *29*: 481–493 (1967).

11 GROSS, R. T. and HURWITZ, R. E.: The pentose phosphate pathway in human erythrocytes. Relationship between the age of the subject and enzyme activity. Pediatrics *22*: 453–460 (1958).

12 GROSS, R. T.; SCHROEDER, E. A. R., and BROUNSTEIN, S. A.: *Energy metabolism* in the erythrocytes of premature infants compared to full term newborn infants and adults. Blood *21*: 755–763 (1963).

13 HASLAM, R. J. and MILLS, D. C. B.: The adenylate kinase of human plasma, *erythrocytes and platelets in relation to the degradation of adenosine diphosphate* in plasma. Biochem. J. *103*: 773–784 (1967).

14 JONES, P. E. H. and MCCANCE, R. A.: Enzyme activities in the blood of infants and adults. Biochem. J. *45*: 464–467 (1949).

15 KOUTRAS, G. A.; HATTORI, M.; SCHNEIDER, A. S.; EBAUGH, F. G., Jr., and VALENTINE, W. N.: Studies on chromated erythrocytes. Effect of sodium chromate on erythrocyte glutathione reductase. J. clin. Invest. *43*: 323–331 (1964).

16 LACHHEIN, L.; GRUBE, E.; JOHNIGK, C. und MATTHIES, H.: Der Verbrauch an Glucose, Galaktose, Ribose und Inosin von Erwachsenen- und Nabelschnur-Erythrocyten. Klin. Wschr. *39*: 875 (1961).

17 MARKS, P. A.; JOHNSON, A. B., and HIRSCHBERG, E.: Effect of age on enzyme activity in erythrocytes. Proc. nat. Acad. Sci., Wash. *44*: 529–536 (1958).

18 MICHEL, H. O.: An electrometric method for the determination of red blood cell and plasma cholinesterase activity. J. Lab. clin. Med. *34*: 1564–1568 (1949).

19 MIWA, S.; TANAKA, K. R., and VALENTINE, W. N.: Erythrocytic and leukocytic glycolytic enzyme studies in hematologic and nonhematologic diseases. Acta haemat. jap. *25*: 12–26 (1962).

20 NECHELES, T. F.; BOLES, T. A., and ALLEN, D. M.: Erythrocyte glutathione-peroxidase deficiency and hemolytic disease of the newborn infant. J. Pediat. *72*: 319–324 (1968).

21 OSKI, F. A. and NAIMAN, J. L.: Red cell metabolism in the premature infant. I Adenosine triphosphate levels, adenosine triphosphate stability and glucose consumption. Pediatrics *36*: 104–112 (1965).

22 OSKI, F. A. and NAIMAN, J. L.: Hematologic problems in the newborn (Saunders, Philadelphia 1966).

23 OSKI, F. A.: Red cell metabolism *in the newborn infant. V. Glycolytic intermediates* and glycolytic enzymes. Pediatrics *44*: 84–91 (1969).

24 OSKI, F. A. and SMITH, C.: Red cell metabolism in the premature infant. III. Apparent inappropriate glucose consumption for cell age. Pediatrics *41*: 473–482 (1968).

25 PAGLIA, D. E. and VALENTINE, W. N.: Studies of the quantitative and qualitative characteristics of erythrocyte glutathione peroxidase. J. Lab. clin. Med. *70*: 158–169 (1967).

26 SASS, M. D.; VORSANGER, E., and SPEAR, P. W.: Enzyme activity as an indicator of red cell age. Clin. chim. Acta *10*: 21–26 (1964).

27 SCHNEIDER, A. S., VALENTINE, W. N., HATTORI, M., and HEINS, H. L., jr.. Hereditary hemolytic anemia with triosephosphate isomerase deficiency New Engl. J Med. 272 229–235 (1965).

28 STAVE, U und CARA, J.. Adenosinphosphate im Blut Frühgeborener Biol. Neonat. 3 160–173 (1961).

29 STAVE, U und HINTZ, H.. Über die Erythrocyten der Frühgeborenen. Hämatologische und enzymologische Studien im ersten Lebensjahr Z. Kinderheilk. 86 184–197 (1961)

30 STEWART, A. G and BIRBECK, J A.. The activities of lactate dehydrogenase, transaminase, and glucose-6-phosphate dehydrogenase in the erythrocytes and plasma of newborn infants. J Pediat. 61 395–404 (1962)

31 SWIERCZEWSKI, E., GIBELIN, C. et MINKOWSKI, A.. Glutathion dans les globules rouges de la veine ombilicale comparaison avec le glutathion dans les globules rouges de la femme enceinte. Biol. Neonat. 3 321–329 (1961)

32 TANAKA, K. R., VALENTINE, W N., and MIWA, S.. Pyruvate kinase (PK) deficiency hereditary non-spherocytic hemolytic anemia. Blood 19 267–295 (1962).

33 VALENTINE, W N., HSIEH, H, PAGLIA, D E., ANDERSON, H M., BAUGHAN, M. A., JAFFÉ, E. R., and GARSON, O M.. Hereditary hemolytic anemia associated with phosphoglycerate kinase deficiency in erythrocytes and leukocytes. A probable X-chromosome-linked syndrome. New Engl. J Med. 280 528–534 (1969).

34 VALENTINE, W N., KONRAD P N., and PAGLIA, D E.. Red cell enzymatic abnormalities in dyserythropoietic anemias. Clin. Res. 20 628 (1972)

35 VALENTINE, W N and KÜRSCHNER, K. K.. Studies on human erythrocyte nucleotide metabolism. I Non-isotopic methodologies. Blood 39 (in press, 1972)

36 VALENTINE, W N, OSKI, F A., PAGLIA, D E., BAUGHAN, M A., SCHNEIDER, A. S., and NAIMAN, J L.. Hereditary hemolytic anemia with hexokinase deficiency Role of hexokinase in erythrocyte aging. New Engl. J Med. 276 1–11 (1967)

37 VALENTINE, W N., PAGLIA, D E., NEERHOUT, R. C., and KONRAD P N.. Erythrocyte glyoxalase II deficiency with coincidental hereditary elliptocytosis. Blood 36 797–808 (1970)

38 WHAUN, J M and OSKI, F A.. Relation of red blood cell glutathione peroxidase to neonatal jaundice J Pediat. 76 555–560 (1970)

39 WITT, I., HERDAN M und KÜNZER, W.. Vergleichende Untersuchungen von Enzymaktivitaten in Reticulocyten reichen und Reticulocyten-armen Fraktionen aus Neugeborenen und Erwachsenenblut. Klin. Wschr 45 149–151 (1968).

40 WITT, I., MÜLLER, H. und KÜNZER, W.. Vergleichende biochemische Untersuchungen an Erythrocyten aus Neugeborenen- und Erwachsenen Blut. Klin. Wschr 45 262–264 (1967).

41 ZINKHAM, W H.. An *in vitro* abnormality of glutathione metabolism in erythrocytes from normal newborns. Mechanism and clinical significance. Pediatrics 23 18–32 (1959)

Authors' address PATRICIA N KONRAD, MD, Assistant Professor of Pediatrics, WILLIAM N VALENTINE, MD, Professor of Medicine, and DONALD E. PAGLIA, MD, Assistant Professor of Pathology, Center for the Health Sciences, University of California Los Angeles, *Los Angeles CA 90024* (USA)

Acta Haemat. 48: 292–296 (1972)

The *in vitro* Incorporation of (2-^{14}C) Glycine into Glutathione of Human Red Blood Cells and the Formation of a Haemoglobin-Glutathione Complex

J. L. Zaidman

Institute of Chemical Pathology, Government Hospital, Tel Hashomer

Abstract. The *in vitro* formation of radioactive disulphides within the intact red blood cells incubated with (2-^{14}C) glycine, is described. Hb A_3 and Hb A_1 fractions separated by electrophoresis were found to contain bound radioactive glutathione. Fraction Hb A_3 was found to contain relatively more bound radioactive glutathione (7 fold) than fraction Hb A_1 if the ratio Hb A_3 to Hb A_1 (1:12) is taken into account.

Key Words
Erythrocyte glutathione
Glutathione in haemoglobin
Glycine incorporation in glutathione
Haemoglobin formation
Haemoglobin A fractions

It has been demonstrated [4] that whole blood and haemolysates are capable to incorporate 2-^{14}C glycine into their glutathione (G-SH), and that the red blood cells are capable to build *de novo* G-SH. G-SH has been found bound to 2 fractions obtained from haemolysates, by electrophoresis, both containing haemoglobin [5]. In a previous report [7] we described the *in vitro* formation of mixed disulphides of glutathione and various Hb A_1 minor components (as demonstrated by Amberlite CG 50 column chromatography).

This paper describes our findings on the *in vitro* formation of radioactive G-SH bound to haemoglobin in mixed disulphide bond, within the intact red blood cells incubated with (2-^{14}C) glycine.

Materials and Methods

Freshly heparinized venous blood samples were collected from healthy normal adults.

Incubations with (2-^{14}C) glycine were based on the method of HOCHBERG [4] 10 ml of whole blood were centrifuged for 10 min at 3 000 rpm 4 ml of plasma were substituted by 4 ml isotonic phosphate buffer, 310 m Osm, pH 7 4 The blood was incubated, at 37 °C, for 20 h with a solution of (2 ^{14}C) glycine containing 50 mCi/10 mmole

Haemolysates and oxyhaemoglobin from blood taken before and after incubation, were prepared as previously described [6] The red cells were washed with saline and lyzed with water and toluene Oxyhaemoglobin was prepared by equilibrating the stroma free haemolysates in a tonometer A part of the haemolysates was dialyzed

Amberlite CG 50 column chromatography of 3.5 ml dialyzed haemolysate was performed according to ZAIDMAN [6 7] The 8 haemoglobin components were collected in 4 fractions fraction 1 contained components A_{1a+b} fraction 2 contained components A_{1c+d} fraction 3 contained component A_{1e} fraction 4 contained components $A_1+A_2+A_{111a}$[1] The different fractions were lyophilized and resuspended with 1.8-3.5 ml bidistilled water

High voltage electrophoresis for amino acid separation from haemoglobin and for haemoglobin separation from glycine was carried out for 3 and 4 h respectively, according to the methods of HOCHBERG *et al* [3] and HOCHBERG [4] on sheets of 52 × 20 cm of Whatman paper No 3 Amino acids were detected with ninhydrin.

Electrophoresis on Cellogel strips was performed by the methods of DIOGUARDI [1] and GRAHAM and GRUNBAUM [2] By this method 6 fractions are separated from haemolysate 4 of haemoglobin UC_1 A_3 A_2 and A_1 and 2 non haemoglobin fractions UC_1 and UC_2

The specific radioactivity of the disulphides in the haemolysates, different fractions and spots separated by electrophoresis was counted as described by HOCHBERG [4]

Results

The radioactivity of the different haemoglobin fractions is summarized in table I Approximately 10% of the radioactive glycine is found in components which cannot be dialyzed About 90% of the radioactivity which remained in the haemolysate after dialysis can be measured in the 4 fractions as separated by column chromatography

In order to ensure that the radioactivity found in the *different fractions* is bound to haemoglobin and not to any other component with a identical chromatographic mobility as haemoglobin, equal volumes of the different fractions were separated by high voltage electrophoresis, for separation of haemoglobin from amino acids After electrophoresis the different haemoglobin spots were cut of and counted The ratio between the total ra-

[1] A_{111a}—a supplementary unknown component obtained only by Amberlite CG 50 column chromatography

Table I. The radioactive activity of haemoglobin from normal human red blood cells. The various haemoglobin components were separated by Amberlite CG 50 column chromatography

Haemolysate or fraction	Volume of haemolysates or fractions ml	Counts/min/ml haemolysate or fraction	Counts/min/ml entire haemolysate or fraction	Counts/min/spot[1]
Haemolysate nondialyzed	3.5	300 000	1 050 000	
Haemolysate dialyzed	3.5	33 000	115 000	650
Fraction 1 A_{Ia+b}	2.0	23 600	47,200	160
Fraction 2 A_{Ic} + A_{Id}	1.8	5 000	9 000	43
Fraction 3 A_{Ie}	3.0	2,180	6,550	29
Fraction 4 A_1 + A_2 + A_{IIIa}[2]	3.0	12,500	37,500	150

[1] The spots of haemoglobin obtained by high voltage electrophoresis of different fractions.

[2] A_{IIIa} = a supplementary unknown component, obtained only by Amberlite CG 50 column chromatography.

dioactivity of the different fractions was found to be similar, as measured by the 2 methods.

By the above-mentioned electrophoresis method glycine has an identical electrophoretic mobility as haemoglobin. Therefore, a supplementary electrophoretic separation was performed by the method which separates between them. It was found that approximately 50% of the radioactivity counted in fraction 4 is due to undialyzed glycine.

The specific radioactivities presented in table I show that most of the radioactivity is divided more or less between fractions 1 and 4, but half of it in fraction 4 is due to undialyzed glycine. Taking into consideration that only 7% of Hb A are minor components, it is obviously that the specific radioactivity of this components, especially of A_{Ia+b}, is higher than in all the other haemoglobin components, A_1 and A_2 (93% of the total). The minor A_1 components seem to bind preferentially the radioactive material as compared with the other components.

In order to verify the above-obtained results by another method, the different haemoglobin fractions, obtained by column chromatography, were separated by Cellogel electrophoresis. As can be seen from table II, fraction 1 obtained by column chromatography is heterogeneous and

Table II The electrophoretic separation on Cellogel of the different haemoglobin fractions as obtained by Amberlite CG 50 chromatography

a) Detection for proteins

Fractions	Cellogel components					
	UC_2	UC_1	A_2	A_1	A_3	UC_3
Fraction 1 A_{3a+b}	+	+	–	–	+	+
Fraction 2 $A_{3c}+A_{3d}$	–	+	–	–	+	–
Fraction 3 A_{3e}	–	–	–	–	+	–
Fraction 4 $A_1+A_2+A_{IIIa}$[1]	–	–	+	+	–	–

b) Radioactive activity, cpm/ml

Fractions	Cellogel components					
	UC_2	UC_1	A_2	A_1	A_3	UC_3
Fraction 1 A_{3a+b}	–	–	–	–	1,000	–
Fraction 2 $A_{3c}+A_{3d}$	–	–	–	–	300	–
Fraction 3 A_{3e}	–	–	–	–	250	–
Fraction 4 $A_1+A_2+A_{IIIa}$[1]	–	–	100	500	100	–

[1] Supplementary unknown component, obtained only by Amberlite CG 50 column chromatography

contains fractions UC_3, A_3, UC_1 and UC_2 but the entire radioactivity was found only in A_3. Fraction 2 contains UC_3 and A_3, and fraction 3 contains only fraction A_3. The entire radioactivity of fractions 2 and 3 is found only in A_3. Most of the radioactivity in fraction 4 is found in A_1.

Discussion

Our findings show that after incubation of human whole blood with radioactive glycine and phosphate the specific radioactivity is found in the haemoglobin fractions A_3 and A_1. In a previous paper [7] we demonstrated the formation of mixed disulphides of glutathione and haemoglobin. We can, therefore, assume that the radioactivity found in the above-mentioned fractions is due to bound radioactive glutathione. Fraction Hb

A_2 was found to contain relatively more bound radioactive glutathione (7-fold) than fraction A_1 if the ratio A_2 to A_1 (1·12) is taken into account

Acknowledgements. We wish to express our thanks to Prof. A. DEFANT (Hebrew University, Jerusalem) and to Prof. A. SZEINBERG (Tel Aviv University Medical School) for their interest and encouragement.

References

1 DIOCLARDI, N. Improved method for electrophoretic separation of the haemoglobin on Cellogel (Chemetron, Milano 1964)
2 GRAHAM, J. L. and GRUNBAUM, B. W. A rapid method for microelectrophoresis and quantitation of haemoglobins on cellulose a state. Amer. J. clin. Path. 39: 567 (1963)
3 HOCHBERG, A., RICHT, M., and DIMANT, E. The incorporation *in vitro* of glycine and L-glutamic acid into glutathione of human erythrocytes. Biochim. biophys. Acta 90: 464 (1964)
4 HOCHBERG, A. Glutathione metabolism in human blood, thesis submitted for the degree of Doctor of Philosophy to the senate of the Hebrew University, 1966.
5 NIV, Y., HOCHBERG, A., and DIMANT, E. The metabolism of glutathione of human blood. Biochim. biophys. Acta 127: 26 (1966)
6 ZAIDMAN, J. L. On the heterogeneity of hemoglobin from normal and glucose-6-phosphate dehydrogenase deficient individuals. Clin. chim. Acta 23: 67 (1969)
7 ZAIDMAN, J. L. Effect of oxidized glutathione and acetylphenylhydrazine on haemoglobin from normal and glucose-6-phosphate dehydrogenase deficient individuals. Brit. J. Haemat. 19: 237 (1970)

Author's address: Dr. JACOB L. ZAIDMAN, Institute of Pathological Biochemistry, Asaf Harofe Government Hospital, Tel Aviv University Medical School, Zerifin (Israel)

Acta Haemat. 48 207-212 (1972)

Binding of Folic Acid to Human Plasma Proteins

L. ELSBORG

University Department of Medicine II (Haematology)
and Department of Clinical Chemistry, Aarhus Amtssygehus Aarhus

Abstract On the basis of ultrafiltration experiments, it is demonstrated that the binding of folic acid to plasma depends on pH When pH is increased, plasma protein binding of folic acid decreases At pH 7 4, approximately 50% of plasma folic acid is bound to plasma proteins, predominantly albumin. No carrier protein other than albumin was detected The binding of folic acid to plasma proteins is not reduced by barbital sodium. Paper electrophoresis was found unreliable in the investigation of folic acid binding to plasma proteins

Key Words
Barbiturates and folic acid
Folic acid and plasma pH
Folic acid in plasma
Ultrafiltration

The folic acid present in plasma is partly freely diffusible, partly combined with plasma proteins [4] By ultrafiltration of plasma, it was found that the free fraction amounts to 35-45% of the total plasma folic acid concentration [7] A somewhat larger proportion of folic acid (60%) was found outside the protein band in Sephadex gel filtration experiments [6], while almost none of the folic acid was found combined to plasma proteins in experiments with electrophoretic separation of plasma samples [2,7]

Barbital sodium is believed to alter the protein binding of folic acid in plasma, causing folic acid deficiency during anticonvulsant drug therapy [5]

The present study shows that folic acid binding to plasma proteins depends on pH At pH 7 4, approximately 50% of plasma folic acid is ultrafiltrable through Millipore filters*. If barbital sodium is added to plasma, the relation between the combined and the uncombined fraction of folic acid does not change

Materials and Methods

Ultrafiltration of plasma samples Tritiated folic acid is added to pooled human plasma samples (protein concentration 70 g/l) in concentrations of 5 ng/ml to

10 000 ng/ml. The activity was 1 μCi/ml in all samples. pH was adjusted to 7.4 by a gas mixture (95% O_2 + 5% CO_2). The samples were ultrafiltrated through 25 nm Millipore filters* under a pressure of 2 atm. By gas proportional counting [3] the activity of tritium in the ultrafiltrate was determined in comparison to the activity in the original plasma samples. The protein bound fraction of folic acid was calculated as

$$\left(1-\frac{^{3}H\text{-activity per volume in ultrafiltrate}}{^{3}H\text{-activity per volume in plasma}}\right)\times 100\%.$$

In 10 experiments using plasma admixed ^{131}I labelled albumin, it was found that 7.5 ± 3.8% of ^{131}I was ultrafiltrated, whereas the protein concentration in the ultrafiltrate amounted to 3.1 ± 1.3% of the protein concentration in the original plasma sample. As the filter is thus highly effective in withholding protein particles, no correction for filter efficiency was made in the following experiments.

Electrophoresis of plasma samples. Paper electrophoresis of plasma samples admixed tritium labelled folic acid in a concentration of 5 ng/ml was carried out in barbituric buffer, pH 8.6, for 18 h. The activity of tritium was measured in each of the plasma protein fractions and is given as a percentage of the total amount of activity started.

Results

In ultrafiltration experiments (fig. 1) it was found that approximately 55% of folic acid in plasma was non-filtrable throughout a very broad range of concentrations. Even at concentrations 1,000 times larger than the normal concentration of folic acid in plasma, there is no change in the relation between the filtrable and the non-filtrable fraction of folic acid.

With folic acid dissolved in a bicarbonate buffer, pH 7.4, throughout the same concentration range as above, it was found that only 5% of the folic acid was retained by the filter itself, probably by adsorption to the filter (fig. 1). If this figure is used as a 'blind value', the protein bound fraction of folic acid in plasma amounts to approximately 50% of the total concentration of folic acid.

Ultrafiltration of plasma samples adjusted to various pH (fig. 2) showed that a rise of pH in plasma resulted in decreasing folic acid binding from 70% at pH 5.1 to 44% at pH 8.6.

By the same technique, the folic acid binding to plasma proteins was studied in plasma samples to which barbital sodium was added at a concentration of 100 mg/l. This is well above the usual level for barbiturate concentrations during anticonvulsant drug therapy. Figure 3 shows that the fraction of folic acid bound to plasma protein is unaltered by barbital sodium over a large concentration range of folic acid.

To human albumin (Kabi®, 5%) folic acid was added in concentra-

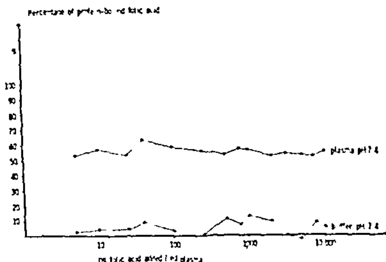


Fig 1 B nd ng of folic acid to plasma proteins. Ultrafiltration under 2 atm pressure.

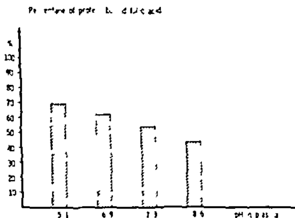


Fig 2 Binding of folic acid to plasma proteins at various pH Ultrafiltration under 2 atm pressure

tions from 10 to 1,000 ng ml After ultrafiltration, it was found that approximately 48% (43% after correction for blind value) of total folic acid concentration was bound to albumin in the concentration range investigated (fig 4)

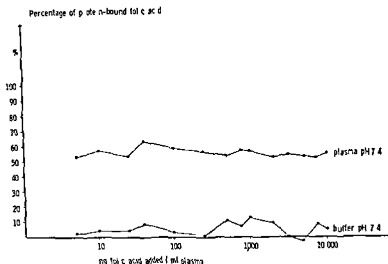


Fig 1 Binding of folic acid to plasma proteins Ultrafiltration under 2 atm pressure

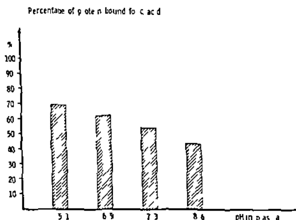


Fig 2 Binding of folic acid to plasma proteins at various pH Ultrafiltration under 2 atm pressure

tions from 10 to 1,000 ng/ml After ultrafiltration, it was found that approximately 48% (43% after correction for blind value) of total folic acid concentration was bound to albumin in the concentration range investigated (fig 4)

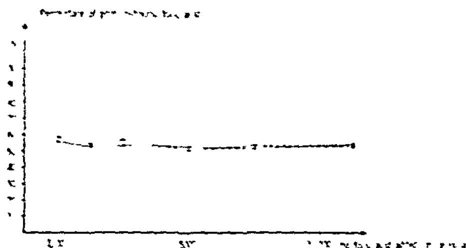


Fig. 3. Effect of barbital sodium on binding of folic acid to plasma proteins. ●——● Plasma. ●——● Plasma with barbital sodium 100 mg/l.

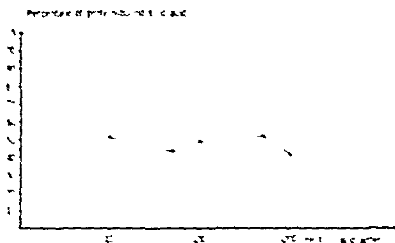


Fig. 4. Binding of folic acid to human albumin 5%. Ultrafiltration under 2 atm pressure.

Paper electrophoretic separation (pH 8.6) of plasma protein samples admixed tritium-labelled folic acid (fig. 5) showed that folic acid moved faster than albumin and only a slight amount of folic acid was found in the protein bands. Equal mobility of folic acid was found by electrophoresis in a buffer solution, pH 8.6, without protein containing tritium labelled folic acid. When electrophoresis was carried out at pH 7.2, however, folic acid was predominantly found in the albumin band.

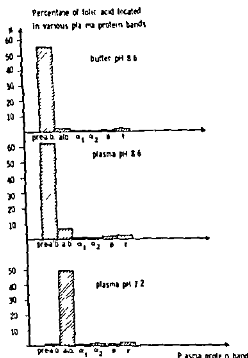


Fig 5 Mobility of folic acid on electrophoresis at pH 8 6 and 7 2 for 18 h.

Discussion

In the concentration range of 10^{-8} to 10^{-4} mole/l, approximately 50% of the folic acid in plasma was non filtrable bound to plasma protein This result is in agreement with results obtained by JOHNS *et al* [4], NEAL and WILLIAMS [7] and ALTER *et al* [1] The proportion between bound and unbound folic acid depends on pH, the free fraction of folic acid decreasing when pH increases This suggests that the binding results from an interaction of ionized groups of folic acid with corresponding groups of plasma proteins Ultrafiltration of pure solutions of human albumin at pH 7.5 gave a binding pattern for folic acid similar to plasma On the average, binding was 7% less, suggesting that only small amounts of folic acid are bound to proteins other than albumin No difference in the proportion between bound and unbound folic acid was detected by ul-

trafiltration of plasma with small and large folic acid concentrations. It is therefore unlikely that a small specific protein fraction (carrier protein) exists to which folic acid is bound with a higher affinity than to albumin.

The electrophoretic mobility of folic acid at pH 8.6 was faster than albumin. By paper electrophoresis, plasma and folic acid, however, are subjected to non-physiological conditions, which might change the association constant for folic acid, i.e. the pH in the barbiturate buffer will tend to reduce the binding of folic acid to plasma (fig. 2). When electrophoresis was carried out at pH 7.2 in a phosphate buffer, mobility was the same for folic acid and albumin. This supports the hypothesis that folic acid is bound to albumin, but is by no means conclusive as the mobility of folic acid was the same by electrophoresis in a buffer solution and in plasma at pH 8.6. Paper electrophoresis is presumably unreliable for the investigation of folic acid binding to plasma proteins.

Displacement of folic acid from a carrier plasma protein has been suggested by KLIPSTEIN [5] as a possible explanation for folic acid deficiency occurring during anticonvulsant therapy with barbiturates. In this study, however, no change in folic acid binding to plasma proteins was detected after addition of a barbiturate (barbital sodium) to the plasma samples.

References

1 ALTER, H. J.; ZVAIFLER, N. J., and RATH, C. E.: Interrelationship of rheumatoid arthritis, folic acid and aspirin. Blood *38*: 405 (1971).

2 CONDIT, P. T. and GROB, D.: Observation on the metabolism of folic acid in man and effect of aminopterin. Cancer, Philad. *11*: 525 (1958).

3 ELSBORG, L.: *In press*, 1972.

4 JOHNS, D. G.; SPERTI, S., and BURGEN, A. S. V.: The metabolism of tritiated folic acid in man. J. clin. Invest. *40*: 1684 (1961).

5 KLIPSTEIN, F. A.: Subnormal folate and macrocytosis associated with anticonvulsant drug therapy. Blood *23*: 68 (1964).

6 MARKKANEN, T. and PELTOLA, O.: Binding of folic acid activity by body fluids. Acta haemat., Basel *43*: 272 (1970).

7 NEAL, G. E. and WILLIAMS, D. C.: The fate of intravenously injected folate in rats. Biochem. Pharmacol. *14*: 903 (1965).

Author's address: Dr. L. ELSBORG, University Department of Medicine II (Haematology), Aarhus Amtssygehus, *DK-8000 Aarhus C* (Denmark); requests for reprints to Prof. P. BASTRUP MADSEN, University Department of Medicine II (Haematology), Aarhus Amtssygehus, *DK-8000 Aarhus C* (Denmark).

Acta Haemat 48 213-217 (1972)

Transferrin, the Third Carrier Protein of Folic Acid Activity in Human Serum

T Markkanen, S Virtanen, P Himanen and R -L. Pajula

Department of Medical Microbiology, University of Turku Turku

Abstract Serum proteins were divided into protein groups by DEAE Sephadex A 50 gel chromatography One of the proteins to be identified had gone toward the cathode and contained no folic acid activity The other went in the electrical field toward the anode and contained all the folic acid activity applied This protein proved to be transferrin

Key Words
Carrier proteins
Folic acid metabolism
Gel chromatography
Transferrin

It has earlier been reported from our laboratory that, in Sephadex gel chromatography, some 30–40% of the folic acid activity (FAA) of the serum is bound to serum proteins and the balance is eluated 'free' after the separation of proteins [4–7] It is possible, however, that some of the FAA bound to proteins *in vivo* is split off during chromatography The FAA is so firmly bound to proteins that the bonds withstand relatively strong treatment, e g ammonium sulphate precipitation and redissolution Bound FAA is also secreted into the urine in the nephrotic syndrome and is then chromatographically demonstrable [6]

In an analysis of the FAA component bound to proteins, it was noted at the outset, that some was bound to serum albumin [5] The albumin bound FAA was about one fifth of all the protein bound FAA [7] The result could be reproduced and confirmed When the analysis of the protein bound FAA was continued it was seen that the majority, some 40–50%, in DEAE Sephadex chromatography was bound to α-2-macroglobulin [7] This finding also proved to be constant and reproducible

The following is a report of a study which showed how a third FAA carrier protein could be purified and identified from the serum

Material and Methods

The taking and treatment of serum samples has been described in detail in an earlier paper [7]. In the primary isolation of the protein groups we used the DEAE Sephadex A 50 chromatography as described in detail in an earlier paper [7]. The FAA of the fractions obtained was determined microbiologically by the *L. casei* method as described before [4]. After the FAA within the area of each protein group had been ascertained, the present study continued to treat protein group 2 (protein maximum) of figure 1. First, the fractions had to be concentrated until protein amounts sufficient for starch electrophoresis had been accumulated. The fractions already contained such large amounts of FAA that they could easily be demonstrated in the microbiological FAA determination.

After chromatography, the fractions of protein group 2 (fig. 1) were concentrated in the following way: the fractions were pipetted into dialysis bags which were submerged into a dextran T 500-solution (15% in water, Pharmacia, Uppsala) and placed into a refrigerator for 3 days. During this time the excessive electrolytes and the volume increasing fluid were dialyzed from the fractions into the dextran. The fractions were in this way concentrated to about one fifth of their original volume. In detail, the concentration was carried out as described before [2].

After the concentration, the protein components of the fractions were identified in immunoelectrophoresis [10] and by the immunodiffusion test [8], also described in detail previously. The fractions of protein group 2 proved to contain two proteins: γ-globulin and transferrin.

These two proteins were separated in horizontal starch gel electrophoresis using hydrolyzed starch (Connaught Medical Research Laboratories, Toronto) and the discontinuous tris borate buffer system [1]. One plate was stained with amido black 10 B [9]. The part of the unstained plate corresponding to the unidentified protein was cut off for FAA determination. The cut plates were suspended in the phosphate buffer used in the *L. casei* determination and the FAA was determined as described above.

Results

The fractions of protein group 2 (fig. 1) contained 2 proteins: in electrophoresis, the γ-globulin fraction went toward the cathode and transferrin toward the anode. Transferrin proved to bind all the FAA that could be extracted in the electrophoretic band. Consequently, transferrin is the third protein identified to date which carries FAA in the serum. The FAA carried by transferrin constituted about one tenth of the FAA bound to the various serum proteins. It is not yet known whether the binding capacity of transferrin was fully saturated by this amount of FAA.

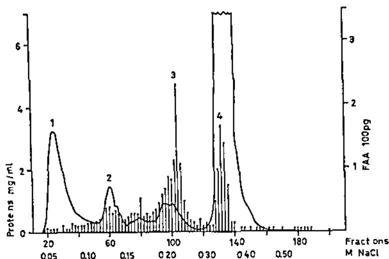


Fig 1 Sephadex DEAE A 50 chromatography of the human serum The protein groups (maxima) are indicated by figures 1–4 The present study dealt with those fractions of protein group 2 that contained γ globulin and transferrin We have shown earlier that the FAA carrier protein of group 3 is α 2 macroglobulin and that of group 4 albumin The FAA of natural serum was 4,100 pg Some 35% of it was bound to the protein area.

Discussion

We have been able to note before that some 40–50% of the total serum FAA is bound to proteins [4–7] This result was obtained after gel chromatography, and it is, therefore, not known whether this state also prevails *in vivo*, or whether chromatography splits off the FAA which may perhaps be loosely bound to serum proteins Continued studies showed that the main FAA carrier protein in the serum was the α-2-macroglobulin [7] This protein seems to bind some 40–50% of the FAA of the protein area Another protein which also carries serum FAA is albumin [5] Its share seems to vary but is probably about 20–30% of all bound FAA Certain clinical pathological conditions can probably affect this vary part of FAA binding, as our preliminary findings seem to suggest [6] The remainder of the protein bound FAA travels in electrophoresis with the transferrin, as the present study now showed

It is known that serum albumin can bind many different substances such as anions fatty acids bilirubin pigments etc [3] The function of the α 2 macroglobulin is partly obscure but it is known to possess carrier protein qualities [3] It is interesting to note that the majority of the FAA bound to serum proteins is attached to this protein One of the clinical diseases in which the amount of this protein is increased is the nephrotic syndrome [3] As pointed out previously relatively large amounts of protein bound FAA are excreted into the urine in this disease [6] So far however it is not known whether all this FAA is bound to *α 2 macroglobulin*

Transferrin is known to bind iron and also to some extent copper [3] Both these metals are important e g for hematopoiesis and in this connection it should be noted that FAA also is bound to transferrin Transferrin has apparently not been found to possess qualities of binding substances other than iron and copper In the electric field this protein is however divided into separate fractions which are genetically important In this early phase we have tried to ascertain whether the FAA is bound to the so-called main bulk component of transferrin or rather to the genetic groups It was observed however that when serum transferrin was treated in a high powered electric field the FAA bound by it disappeared It is possible that when the strength of the electric current was increased the bound FAA was released and raced ahead of the protein components on the starch plate As a result it was not determinable in the cut strips It is also possible that the electric current decomposed the pteroylglutamic acid into components which were no longer biologically active in the *L. casei* determination It could be seen however that in a weak electric current the FAA still travelled with the undivided transferrin but after the strength of the current was increased so that the genetic protein fractions emerged no more FAA could be obtained from any transferrin fraction Methodical future studies will probably show which of the above alternatives in the first place enter into question *Transferrin synthesis apparently takes place in the liver* [3] In later studies it will also be interesting to see the role of the various liver diseases from the point of view of the transferrin FAA binding

References

1 BECKMAN L and HOLMGREN G Transferrin variants in Lapps and Swedes Acta genet Basel *11* 106 (1961)

Acta Haemat. 48: 218–226 (1972)

Fine Structure of the Bone Marrow Sinusoidal Wall in Idiopathic and Drug-Induced Panmyelopathy

J. P. SAMSOM, C. E. HULSTAERT, I. MOLENAAR and H. O. NIEWEG

Division of Hematology, Department of Medicine,
and Center for Medical Electron Microscopy, University of Groningen, Groningen

Abstract. Ultrastructural studies of the bone marrow sinusoids showed no essential difference between the sinusoidal wall of patients suffering from panmyelopathy – idiopathic or drug induced – and from control subjects. No sign of immunological damage was seen. The hypothesis of KNOSPE and CROSBY about the central role of damage to the microcirculation in this disease could not be confirmed. The results are consistent with the alternative possibility that the injury primarily involves the blood cell precursors.

Key Words
Bone marrow sinusoids
Electron microscopy
Panmyelopathy

Acquired panmyelopathy (aplastic anemia) may be induced by various factors such as exposure to some chemicals and drugs [15] and infectious agents, e.g. hepatitis virus [8]. When it is impossible to incriminate an exogenous agent, the disease is classified as idiopathic. This term is applicable in about one half of all cases [15, 17].

Panmyelopathy is usually thought to be the result of stem cell failure either in its dividing or in its differentiating capacities. However, recently KNOSPE and CROSBY [7] have speculated that a defect in the bone marrow microcirculation was the primary cause of the bone marrow damage. They did not perform ultrastructural studies in human disease but presented some evidence based on investigations on irradiated experimental animals in support of this hypothesis. In studies of human panmyelopathy of our group [10] it has been demonstrated that an allergic mechanism is operative in the development of bone marrow damage: abnormal antibody activity was detected both in panmyelopathy secondary to drugs and in idiopathic bone marrow disease.

Possibly an immunological injury to the bone marrow sinusoidal wall could, therefore, be an important factor in the pathogenesis of panmyelopathy. For this reason it was decided to perform an electron microscopic study of the bone marrow sinusoids in human panmyelopathy, both of the idiopathic and the drug induced type.

Patients Studied

The criteria for selecting the patients were those generally accepted [15, 17], anemia, leukopenia and thrombocytopenia without splenomegaly were present in all cases. Examination of sections and smears from the bone marrow with the light microscope showed a hypocellular marrow in all cases. Some data of the patients are given in table I. From the clinical history of the 9 patients studied we were able to find a possible exogenous factor (drugs) in 3 cases, these were classified as secondary and the other 6 cases as idiopathic. Other causes of panmyelopathy could be excluded. Sternal marrow biopsies from 4 patients having a normal bone marrow as assessed by light microscopy served as controls.

Materials and Methods for Electron Microscopy

Bone marrow was obtained by sternal aspiration from 9 patients suffering from idiopathic or drug induced panmyelopathy and from 4 control patients. Within 20 sec after sternal aspiration the bone marrow fragments were fixed in 2% phosphate buffered glutaraldehyde pH 7.35 for 2 h at 4 °C. After washing in phosphate sucrose buffer for 24 h the marrow fragments were postfixed in 2% OsO_4 for 2 h.

Table I

Patient	Age, years	Sex	Etiology	Histology
1	37	M	secondary	large amounts of analgetics and iodochlorhydroxyquin
2	63	F	secondary	phenylbutazon, chloramphenicol, acetylsalicylic acid, APC and indomethacin
3	56	M	idiopathic	
4	17	F	idiopathic	
5	43	F	secondary	periodical panthrol, APC
6	80	M	idiopathic	
7	20	M	idiopathic	
8	75	F	idiopathic	
9	31	F	idiopathic	


FG

This was followed by dehydration in an alcohol propylene oxide series The marrow pieces were then embedded in Epon 812 [9] 1 μm thick sections, stained with toluidine blue, were examined with the light microscope to select the areas containing sinusoids (fig 1) Ultrathin sections were stained with uranylacetate and lead citrate, or silver methenamine They were studied and electron micrographs were taken with an EM 300 Philips electron microscope

Results

After scrutinizing the 1 μm thick sections with the light microscope, several sinusoids were found in the bone marrow of patients and control subjects (fig 1) Very often the sinusoidal content stained slightly more than the interstitial part of the marrow Large fat accumulations were prominent, as usual in bone marrow failure The cell content of all patient bone marrow samples turned out to be very low

Figure 2 shows a low magnification electron micrograph containing a bone marrow sinusoid from a control subject. The thin sinusoidal lining is interrupted in some places The cytoplasm of the sinusoidal lining cell contains several lysosomes

Figure 3 and 4 show electron micrographs from sinusoids of 2 patients It is obvious that the preparation has damaged some of the depicted cells In some places the endothelial lining is interrupted (fig 3) Intercellular junctions between the endothelial cells could be found (fig 3 and inset a and b) Figure 4 (inset a), shows part of an endothelial lining cell containing many micropinocytotic vesicles The ultrastructure of the sinusoidal lining cells from the patients is not essentially different from that of the control subjects

Discussion

In ultrastructural studies of the bone marrow in human disease, attention has been paid almost exclusively to the hemopoietic cellular ele-

Fig 1 Bone marrow of patient No 5 (table I) with idiopathic panmyelopathy as seen in a 1 μm thick section with the light microscope The sinusoids are indicated with arrows. FG = Fat globule Toluidine blue, × 578

Fig 2 Sinusoid in bone marrow of control subject No 1 (table I) as seen with the electron microscope Interruptions in the sinusoidal lining are indicated with arrows. L=Lysosome, N=nucleus of endothelial cell, E=erythrocyte Silver methenamine, × 5 600

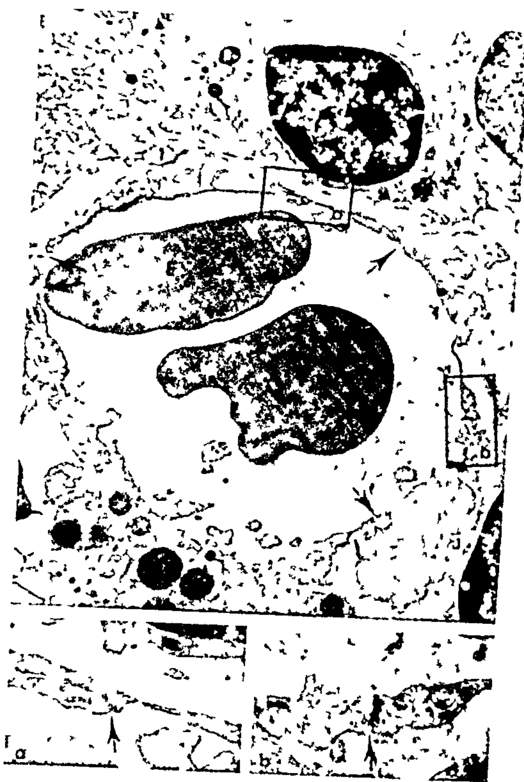


Fig 3

ments The literature concerning the bone marrow vasculature consists mainly of reports of studies of animal tissue [1, 2, 5, 12–14, 18] The sinusoids of the bone marrow differ from comparable vessels of other organs *in their (functionally)* continuous wall and lack of a continuous basement lamina[1] [6] Studies of the bone marrow sinusoids in panmyelopathy are scanty [3, 4]

Concerning a possible defect in the microcirculation in the bone marrow, 3 parameters have to be considered (1) the integrity of the sinusoidal lining cells, (2) the existence of a basement lamina, and (3) the possible occurrence of immune complexes

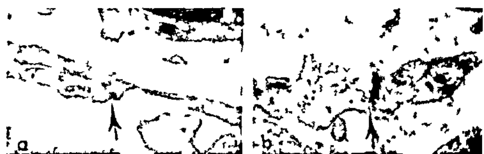
About the normal ultrastructure of the cells constituting the sinusoidal wall, it must be stated that the method of preparation and fixation influences its visualization with the electron microscope The technique of aspiration is liable to cause mechanical damage to the architecture of the bone marrow in particular that of the sinusoidal wall [2, 5] In our electron micrographs the sinusoidal lining is also interrupted in many places To obtain a proper fixation of the endothelial lining of sinusoids, the tissue must be prepared under conditions which cannot be realized when human tissue is fixed Only perfusion fixation, in which the fixation fluid is injected into the vascular system, guarantees an optimal fixation [16] This holds especially for the maintenance of the integrity of structures such as endothelial walls and its fenestration, if present.

Concerning the occurrence of a basement lamina, it must be said that no consensus exists about its continuity according to WEISS [12, 13] it seems to be absent in many places Some authors even think that the lining of the sinusoidal lumen is devoid of a basement lamina [2]

In an evaluation of the possibility that the sinusoids are the target of *the immunological injury in panmyelopathy*, it should be noted that in experimental renal disease induced by antiglomerular antibody the endothelium of the glomerular capillaries is partially or completely replaced by a dense material situated closely to a thin basement lamina In the antigen-antibody complex mediated injury so-called lumpy deposits of electron-dense material are found along the outer aspect of the basement lamina as

[1] The term basement lamina' is used instead of the older term basement membrane

Fig 3 Sinusoid in bone marrow of patient No 5 (table I) The interruptions of the endothelial *lining are indicated with arrows.* × *9,500 Inset* a and *b* depict intercellular junctions (arrows) Lead citrate and uranylacetate, × 27 300


a
b

ments. The literature concerning the bone marrow vasculature consists mainly of reports of studies of animal tissue [1, 2, 5, 12–14, 18]. The sinusoids of the bone marrow differ from comparable vessels of other organs in their (functionally) continuous wall and lack of a continuous basement lamina[1] [6]. Studies of the bone marrow sinusoids in panmyelopathy are scanty [3, 4].

Concerning a possible defect in the microcirculation in the bone marrow, 3 parameters have to be considered: (1) the integrity of the sinusoidal lining cells, (2) the existence of a basement lamina, and (3) the possible occurrence of immune complexes.

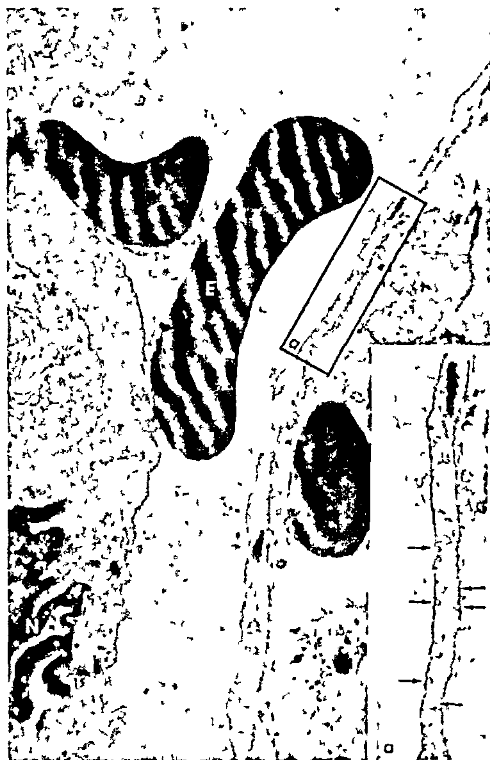
About the *normal* ultrastructure of the cells constituting the sinusoidal wall, it must be stated that the method of preparation and fixation influences its visualization with the electron microscope. The technique of aspiration is liable to cause mechanical damage to the architecture of the bone marrow in particular that of the sinusoidal wall [2, 5]. In our electron micrographs the sinusoidal lining is also interrupted in many places. To obtain a proper fixation of the endothelial lining of sinusoids, the tissue must be prepared under conditions which cannot be realized when human tissue is fixed. Only perfusion fixation, in which the fixation fluid is injected into the vascular system, guarantees an optimal fixation [16]. This holds especially for the maintenance of the integrity of structures such as endothelial walls and its fenestration, if present.

Concerning the occurrence of a basement lamina, it must be said that no consensus exists about its continuity: according to WEISS [12, 13] it seems to be absent in many places. Some authors even think that the lining *of the sinusoidal lumen is devoid of a basement lamina* [2].

In an evaluation of the possibility that the sinusoids are the target of the immunological injury in panmyelopathy, it should be noted that in experimental renal disease induced by antiglomerular antibody the endothelium of the glomerular capillaries is partially or completely replaced by a dense material situated closely to a thin basement lamina. In the antigen-antibody complex mediated injury so-called lumpy deposits of electron-dense material are found along the outer aspect of the basement lamina as

[1] The term 'basement lamina' is used instead of the older term 'basement membrane'.

Fig. 3. Sinusoid in bone marrow of patient No. 5 (table I). The interruptions of the endothelial lining are indicated with arrows. × 9,500. Inset a and b depict intercellular junctions (arrows). Lead citrate and uranylacetate, × 27,300.



F i 4

is also seen in human pathology [11] It is doubtful if one may draw a parallel between glomerulonephritis and a possible humoral or cellular immunological injury to the bone marrow sinusoidal wall [7] The massive glomerular basement lamina, presenting a very good base for deposits of electron-dense material, cannot be compared with the nearly absent and very thin basement lamina of the sinusoidal wall It is, therefore, not surprising that in the electron micrographs no electron-dense deposits resembling those found in the kidney [11] could be detected

From this observation the conclusion that immune complexes are absent cannot be drawn with certainty One should keep in mind that the sample of marrow obtained by sternal aspiration is small and not necessarily representative The immune complexes may not have aggregated to the extent as is the case with the kidney It is also possible that the phagocytic capacity of the sinusoidal cell is responsible for the absence of immune complexes However in our material there is no obvious increase of the number of secondary lysosomes as an indication of a phagocytic process

In this study we did not find an essential difference between the bone marrow sinusoidal wall of the patients with panmyelopathy and that of the control subjects Thus we cannot provide evidence in support of the hypothesis of damage – possibly due to immunological injury – to the microcirculation of the bone marrow as the operative mechanism in panmyelopathy However, these results are consistent with other investigations of our group suggesting that the target of immunological attack is the plasma membrane of the blood cell precursors

Acknowledgements This work was supported by the Netherlands Organization for the Advancement of Pure Research (ZWO), grant No 91 7 The authors thank Miss G de Wit and Mr H R A. Meiboro for skilful technical assistance.

References

1 Bruyn P P H de Thomas, T B and Michelson, S. Fine structure of the vascular components of the guinea pig bone marrow Anat. Rec. 154 [illegible] (1966)

Fig 4 Part of a sinusoid in bone marrow of patient No. 3 (table I). There is an interruption in the endothelial lining. × 9,500 The micropinocytic vesicles can be seen in inset a (arrows) Lead citrate and uranyl acetate, × 22,500

2 BAUYN, P. P. H. DE, MICHELSON, S., and THOMAS, T. B. The migration of blood cells of the bone marrow through the sinusoidal wall. J. Morph. *133* 417–438 (1971).

3 BAYON, P. A., FILAE, D., and RÉVOL, L. Histological and electron microscopic features of bone marrow reticulum and endothelial cells in primary aplastic anemia. *Abstracts 1st Meet. Europ. Div. Int. Soc. Haemat., Milano 1971*

4 BURCKHARDT, R. and DEMMLER, K. The vascularisation of bone marrow in aplastic anemia. Abstracts 1st Meet. Europ. Div. Int. Soc. Haemat., Milano 1971

5 CAMPBELL, F. Fine structure of the bone marrow of chicken and pigeon. J. Morph. *123* 405–441 (1967)

6 HUHN, D. and STICH, W. Blood vessels and nerves, in Fine structure of blood and bone marrow (Lehmanns, Munich 1969)

7 KNOSPE, W. H. and CROSBY, W. Aplastic anemia, a disorder of the bone marrow sinusoidal microcirculation rather than stem-cell failure. Lancet *I* 20–22 (1971)

8 LEVY, R. N. SAVITSKY A., FLORMAN, A. L., and RUBIN, L. Fatal aplastic anemia after hepatitis report of five cases. New Engl. Med. *273* 1118–1123 (1965)

9 LUFT, J. H. Improvement in epoxy resin embedding methods. J. biophys. biochem. Cytol. 9 409–414 (1961)

10 NIEWEG, H. O., SAMSOM, J. P. GAST, G. C. DE, SCHANS, G. C. V.D., and BOS-V. ZWOL, F. Spoiled membrane allergy in the etiology of panmyelopathy (aplastic anaemia) (In press.)

11 UNANUE, E. A., DIXON, F. J., and FELDMAN, J. D. Experimental immunologic diseases of the kidney, in MIESCHER and MÜLLER EBERHARDT Textbook of immunopathology (Grune & Stratton, New York 1968)

12 WEISS, L. An electron microscopic study of the vascular sinuses of the bone marrow of the rabbit. Bull. Johns Hopk. Hosp. *108* 171–178 (1961)

13 WEISS, L. The structure of bone marrow. J. Morph. *117* 467–538 (1965)

14 WEISS, L. Transmural cellular passage in vascular tissues of rat bone marrow. Blood *36* 189–209 (1970)

15 WINTROBE, M. M. Clinical hematology (Lea & Febiger, Philadelphia 1967)

16 WISSE, E. *An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids.* J. Ultrastruct. Res. *31* 125–150 (1970).

17 WIJNJA, G. SNIJDER, J. A. M., and NIEWEG, H. O. Acetylsalicylic acid as a cause of aplastic anemia. Lancet *II* 768–771 (1966)

18 ZAMBONI, L. and PEASE, D. The vascular bed of red bone marrow. J. Ultrastruct. Res. *5* 65–85 (1961)

Authors' addresses: Dr. J. P. SAMSOM and Dr. H. O. NIEWEG, Department of Hematology, Academisch Ziekenhuis, Oostersingel 59, *Groningen*; Dr. C. E. HULSTAERT and Dr. L. MOLENAAR, Center for Medical Electron Microscopy, Oostersingel 69-1, *Groningen* (The Netherlands)

Acta Haemat. 48 227–233 (1972)

Influence of Cell Density on the Acridine Orange Binding to Deoxyribonucleoprotein Complex in Leucocytes from Patients with Infectious Mononucleosis and Acute Leukaemia[1]

L. BOLUND, L. E. DE BAULT, G. E. FOLEY[2], G. GAHRTON, D. KILLANDER, W. C. MOLONEY, R. RIGLER, A. SKARIN and B. WAHREN

Institute for Medical Cell Research and Genetics, Medical Nobel Institute, Karolinska Institutet, and Department of Internal Medicine and Radiumhemmet, Karolinska Sjukhuset, Department of Virology, Statens Bakteriologiska Laboratorium, Stockholm, The Children's Cancer Research Foundation, and Department of Pathology, Harvard Medical School, and Peter Bent Brigham Hospital, Boston, Mass.

Abstract. The acridine orange (AO) binding to the deoxyribonucleoprotein (DNP) complex of individual normal lymphocytes, infectious mononucleosis (IM) lymphoid cells and leukaemic blast cells was determined by microfluorometry. Increased AO binding to DNP was found with increasing cell density on the slide and was similar in the different cell populations. Only in exceptional cases of IM, the AO binding was high irrespective of cell density. The increased AO binding was due to changes in the DNP complex, which might be induced by the release of macromolecular substances from the cells. The results show that in clinical cytological studies, the fluorescence intensities cannot be used for the discrimination between normal and malignant cells.

Key Words
Acridine orange binding
Acute leukaemia
Infectious mononucleosis
Leucocyte nucleoproteins
Microspectrofluorometry
Nucleoprotein staining

The fluorescent basic dye acridine orange (AO) predominantly binds to negatively charged phosphate groups in the nucleoprotein complexes [13]. Previous studies have demonstrated increased binding of AO to the

[1] These studies were supported in part by research grants from the Swedish Cancer Society, The Svenska Läkaresällskapet and The Karolinska Institutets Forskningsfonder to the Institute for Medical Cell Research and Genetics, and by research grants C-6516 from the National Cancer Institute and FR-05526 from the Division of Research Facilities and Resources, National Institutes of Health to The Children's Cancer Research Foundation.

[2] Holds Research Career Award K6-CA 22.150 from the National Cancer Institute, National Institutes of Health.

deoxyribonucleoprotein (DNP) complex of leucocytes derived from patients with infectious mononucleosis (IM) as compared to normal leucocytes [5]. This increased dye binding was interpreted to be due to a structural change in the DNP complex similar to the change which has been detected with a variety of cytochemical techniques in non proliferating cells soon after growth stimulation or nuclear activation [1, 6, 10, 14, 15]. Such DNP changes seem to be involved in the regulation of genome function. However, recent studies have indicated that similar DNP changes can be induced simply by varying the cell density on the substratum. Thus, crowding of hen erythrocytes [4, 11], HeLa cells [4, 12] and human lymphocytes [2, 4] on glass induces increased binding of AO to DNP. However, the biological importance of this 'crowding effect' is not quite clear. In culture, the cells have to 'condition' their environment before growth can be initiated, a process which is enhanced at higher cell densities. When the cells have succeeded in conditioning the culture milieu and, in particular, the substratum, the cytochemical properties of the chromatin change [4, 12] and initiation of proliferation is probably dependent on this change in the chromatin [3, 12].

The purpose of the present investigation was to determine whether cell density factors might be responsible for the observed differences between AO binding to normal and IM leucocytes, and whether similar phenomena apply equally to human leukaemic cells, which exhibit other properties similar to those of cells derived from IM [8, 9].

Material and Methods

Buffy coat leucocytes from 7 patients with IM, one patient with acute leukaemia and 11 normal donors were studied.

Sampling and preparation of cell cultures. 30 ml of venous blood were collected in a tube containing 5 ml of Ringer's solution with heparin in a final concentration of 35 IU/ml. After sedimentation for 2 h at 37 °C, the leucocyte-rich plasma was collected, the cell suspension centrifuged at 500 g for 7 min, the plasma withdrawn, and the cells resuspended in 2 ml of Eagle's minimal essential medium (MEM). The plasma was then recentrifuged at 2 200 g for 20 min to remove the platelets. A series of cell suspensions at different cell densities was prepared using Eagle's MEM supplemented with plasma, to a final concentration of 25%. Each cell suspension was then implanted in plastic Petri dishes (50 mm in diameter) with Bürker hemocytometer slides on the bottom. After incubation of the cultures at 37 °C in 10% CO_2 in air for 1 h, the slides were quickly washed twice in saline, fixed in equal parts of ethanol and acetone for 30 min at room temperature, and stored in the fixative at 4 °C until stained. A series of slides with different cell densities was thus obtained.

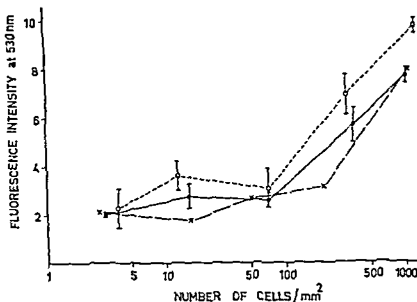


Fig 1 AO binding to DNP (fluorescence intensity at 530 nm) of leucocytes

cases at cell densities between 100 and 500 cells mm²

as indicated in figure 1 The cell density (number of cells per unit surface area) was determined by counting cells within 10 microscopic fields, equally distributed over the whole Burker slide Knowing the cell number inoculated into the Petri dishes, their bottom area and the cell density on the Burker slides after fixation, it was possible to calculate the percentage of cells in the original suspension which was attached to the slides.

AO staining and fluorescence measurements The ethanol/acetone fixed cells were AO-stained as previously described [13] After acetylation with 40% acetic anhydride in pyridine the preparations were stained with 10^{-4} M AO in an Na_2HPO_4 citrate buffer (pH 4 1, ionic strength 0 6), incubated in pure buffer to permit diffusion of unbound dye, and mounted in the citrate buffer and sealed with Entellan (Merck) AO-stained cells were excited individually in UV light (λexcit = 365 nm) and the fluorescence intensities at 530 nm were measured in a microspectrofluorometer as described by CASPERSSON *et al* [7]

AO binds stoichiometrically and specifically to nucleic acids [13, 14] the degree of binding being dependent on the number of available phosphate groups on the nucleic acid, as well as on its secondary structure [13] Nucleic acids with double helical structure (e g. DNA) bind AO in a monomeric form, giving rise to an emission

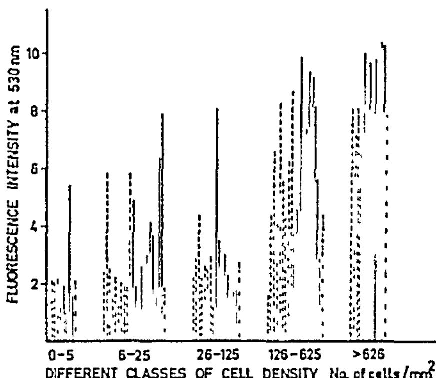


Fig. 2. AO binding to DNP (mean fluorescence intensity at 530 nm of 10–20 measured cells) in leucocytes from normal donors (– – – –), patients with IM (——) and one patient with acute leukaemia (AL) (~~~~) at different cell densities on the slide. AO binding increased at cell densities higher than 125 cells/mm² in most cases. Some individual cases of IM exhibit increased AO binding at low cell densities. Each bar represents one case.

maximum at 530 nm in the microspectrofluorometer used in the present studies, whereas single stranded nucleic acids (e.g. RNA) bind AO in an associated form with an emission maximum at 590 nm [13, 14]. Since the fluorescence intensity at 530 nm (F_{530}) is only slightly dependent on the presence of single stranded nucleic acids, the recorded F_{530} values are a good measure of the amount of AO binding phosphate groups in the DNP complex [13]. Ten to 20 mononuclear leucocytes, distributed over the whole slide, were measured in each sample. The intercellular variation in F_{530} values was quite low, i.e. the coefficient of variation was about 10%. No cells in the G_2 phase of the cell cycle were measured.

Results

Figure 1 indicates that AO binding to the DNP complex of leucocytes, as represented by fluorescence intensity at 530 nm (F_{530}) of individual

Table I Cell adhesiveness to glass slide and AO binding to DNP (F_{530}) in cells derived from IM and in normal leucocytes The F_{530} values are means of 10 cells The F_{530} values of both kinds of cells are increased at the highest cell density There is no clear difference in the degree of adhesiveness to glass between IM and normal cells

Diagnosis	Number of inoculated cells	Number of cells on glass after staining	Percent cells attached on glass	F_{530}
IM	8 640,000	1 024 000	12	7 1
	2,210 000	190,000	9	3 5
	552,500	54 000	10	2.6
	138,100	14,000	10	3 0
Normal	5 600,000	660,000	12	6 6
	1,400,000	160 000	11	2 6
	350 000	20,000	6	1 4
	87,500	8 000	9	1 9
	21,900	4 000	18	2 1

AO-stained cells is highly dependent on the cell density on the glass At low cell densities (less than 100–200 cells/mm²), the F_{530} values are quite constant. Then there is an increase in the degree of AO binding concurrent with increasing cell density A similar type of curve describes this phenomenon in normal, IM and leukaemic leucocytes

As indicated in figure 2, there is a fairly large intercase variation in the AO-binding capacity Some individual cases of IM exhibit a high AO-dye binding, even at low cell densities, which very rarely is the case with control leucocytes. However, the same general pattern of low AO fluorescence at low cell densities and higher AO fluorescence at high cell densities was found with normal, IM, and leukaemic leucocytes

The adhesiveness (to glass) of IM leucocytes and normal leucocytes was similar (table I) The number of cells in the original suspension implanted in the Petri dishes was clearly the most important factor determining the cell density on the glass Thus, the fewer the cells implanted, the lower was the degree of AO binding to the cells

Discussion

The importance of cell density on the slide for the degree of AO binding to nucleoprotein of normal lymphocytes, hen erythrocytes and HeLa

cells has been demonstrated previously [2, 4, 11, 12]. The present studies illustrate a similar increase in the AO binding to the nucleoprotein of leucocytes derived from IM, leukaemia, and normal donors concurrent with increasing cell density. Cells from individual cases of IM, however, exhibit an increased degree of AO binding, even at rather low cell densities.

Under the experimental conditions employed in the present studies, the number of cells plated seems to be the most important factor determining cell density on the glass, differences in adhesiveness to glass being a minor factor. Thus, the explanation for the previously demonstrated increase in AO binding by cells derived from IM may be the implantation of more cells on the slides, since larger numbers of cells were obtained in the buffy coat samples from patients with IM, as compared to those from normal donors.

With respect to the effects of cell density on AO binding, in general, there seemed to be no clear difference between normal lymphocytes, those derived from IM and leukaemic blast cells, with the exception of an occasional individual case of IM which exhibited a high degree of cellular AO binding at low cell densities. The significance of increased AO binding in such cases is uncertain, but it may be related to the proliferative capacity of these cells, or to factors present in the sera of such patients which stimulates cell proliferation, as previously suggested [5]. The most likely explanation for the mechanism by which increased cell density induces increased AO binding is release of a substance (probably macromolecular) from the cells which spreads over the substratum and in some indirect manner affects the state of nuclear DNP [3, 4, 12]. The closer the cells are to each other on the glass, the more effectively this substance conditions the microenvironment and the neighboring cells. In any event, with respect to clinical cytological studies, it is important to note that differences in cell density are crucial factors determining the degree of AO binding, and high fluorescence values in themselves cannot be used for the identification of 'stimulated' lymphocytes or discrimination between normal and malignant cells.

References

1. ANDERSSON, J., KILLANDER, D., MÖLLER, E. and MÖLLER, G. Rapid lymphocyte recognition of histoincompatibility. Changes of the deoxyribonucleoprotein complex in mixed cultures of allogeneic human lymphocytes. J. exp. Med. *131*, 355 (1970).

2 Auer, G., Zetterberg, A., and Killander, D. Changes in binding between DNA and arginine residues in histone induced by cell crowding. Exp. Cell Res. 62 32 (1970).

3 Bolund, L. Cytochemical properties of chromatin and nuclei in relation to gene activation and repression (Tryckeri AB Bader, Stockholm 1971).

4 Bolund, L., Darzynkiewicz, Z., and Ringertz, N. R. Cell concentration and the staining properties of nuclear deoxyribonucleoprotein. Exp. Cell Res. 62 76 (1970).

5 Bolund, L., Gahrton, G., Killander, D., Rigler, R., and Wahren, B. Structural changes in the deoxyribonucleoprotein (DNP) complex of leucocytes from patients with infectious mononucleosis. Blood 35 322 (1970).

6 Bolund, L., Ringertz, N. R., and Harris, H. Changes in the cytochemical properties of erythrocyte nuclei reactivated by cell fusion. J. Cell Sci. 4 71 (1969).

7 Caspersson, T., Lomakka, G., and Rigler, R. Registrierender Fluoreszenzmikrospektrograph zur Bestimmung der Primär- und Sekundärfluoreszenz verschiedener Zellsubstanzen. Acta histochem., suppl. 6, p. 123 (1965).

8 DeBault, L. E., Gahrton, G., and Foley, G. E. Cytochemical populations and [illegible] of cell lines derived from Burkitt's lymphoma, infectious mononucleosis and acute lymphoblastic leukemia. Exp. Cell Res. 65 176 (1971).

9 Gahrton, G. and Foley, G. E. Leukemia [illegible] patterns of the DNA, RNA and protein content of individual mononuclear cells in the peripheral blood of patients with infectious mononucleosis. Cancer Res. 29 1076 (1969).

10 Killander, D. and Rigler, R. Initial changes of deoxyribonucleoprotein and synthesis of nucleic acid in phytohemagglutinin-stimulated human leucocytes in vitro. Exp. Cell Res. 54 163 (1969).

11 Ringertz, N. R. and Bolund, L. Activation of hen erythrocyte deoxyribonucleoprotein. Exp. Cell Res. 55 205 (1969).

12 Ringertz, N. R., Bolund, L., and Darzynkiewicz, Z. Acridine orange binding of intact and [illegible] nuclei in fixed cells in relation to cell growth. Exp. Cell Res. 63 233 (1970).

13 Rigler, R. Microfluorometric characterization of intracellular nucleic acids and nucleoproteins by acridine orange. Acta physiol. scand. 67 suppl. 267 (1966).

14 Rigler, R. and Killander, D. Activation of deoxyribonucleoprotein in human leucocytes stimulated by phytohemagglutinin. II. Structural changes of deoxyribonucleoprotein and synthesis of RNA. Exp. Cell Res. 54 171 (1969).

15 Zetterberg, A. and Auer, G. Changes [illegible] properties of [illegible] [illegible]. Exp. Cell Res. [illegible]

Authors' [illegible]

[illegible] Medicine

Acta Haemat. 48: 234–238 (1972)

A Rapid Screening Test for Thalassaemic Trait[1]

G. Torlontano, A. Tata and A. Camagna

Haematology Unit, Medical Clinic II, University of Rome, Rome,
and Haematology Unit, Department of Medicine, University of Chieti, Chieti

Abstract. Through the study of the volumetric distribution of blood corpuscles conducted with modern electronic equipment, the authors distinguish subjects with β-thalassaemic trait from normal and sideropenic subjects. The investigation described is proposed as a rapid screening test for thalassaemic trait.

Key Words
Coulter counter
Erythrocyte volume
Screening test for thalassaemia
Sideropenia
Thalassaemia

The availability of a screening test for thalassaemic trait is still a very important problem, particularly for those countries with a high incidence of thalassaemia. We feel that this problem may be solved by modern electronic equipment [1–13] providing for a rapid and precise examination of the volumetric distribution of blood cells.

Material and Methods

The Coulter counter model B was used for both the electronic counts and the study of the particle size distribution. This counter applies the principles of electrical gauging to the problem of particle counting [13]. The cells, suspended in an electrically conducting medium, isoton solution (a diluent from the Coulter Elec-

[1] Paper presented to the International Symposium on Standardization in Haematology and Clinical Pathology, S.G. Rotondo (Foggia) 1971.

Supported by a grant from the 'Consiglio Nazionale delle Ricerche' (Special Program TBM).

tronic Sales Co.) flows through a small aperture. The relatively non-conducting cells cause a voltage drop as they pass through the aperture. The resulting amplified pulses are proportional to the size of the cell inducing it. Using increasingly specific instrument threshold settings, each corresponding to definite pulse values starting from a minimal value, it is possible to study the volumetric distribution of blood cells. This study of the volumetric distribution of blood cells was carried out on 13 normal subjects, 16 subjects with β-thalassaemic trait and 10 hyposideraemic patients whole blood samples using d potassium EDTA as the anticoagulant (1 mg/ml) were used.

The haematological study including the evaluation of osmotic fragility of RBC, sideraemia and the percentage of A_2 haemoglobin, was carried out according to standard methods [7].

Results

The characteristic features of the volumetric distribution of normal blood are a high peak near the central zone and a second, slight but clear and constant rise of the curve in the initial left zone (fig. 1). With β thalassaemic subjects, the same investigation always presents the following characteristics (fig. 1): (1) an extremely high peak, clearly shifted toward the left of the curve, (2) total absence of the initial rise in values in the left zone.

Sideropenic patients present the following features (fig. 2): (1) a lower peak than that typical of thalassaemic blood, (2) usually, but not constantly, a slight initial peak.

Discussion

In normal blood the central peak corresponds to erythrocytes of medium volume, which are particularly numerous. The slight initial rise of the curve on the left indicates the presence of platelets in the samples examined [10] and does not appear with platelet free samples.

In thalassaemia, the appearance of the very high maximal peak shifting further toward the left than that of normal samples is attributable to the high number and reduced volume of thalassaemic erythrocytes. The constant absence of the initial rise of the curve on the left with, instead, a steady rise toward the maximal peak, may be attributed to the very small and numerous erythrocytes and schistocytes in thalassaemic blood. These red blood cells or their fragments mix with the platelets, obliterating the

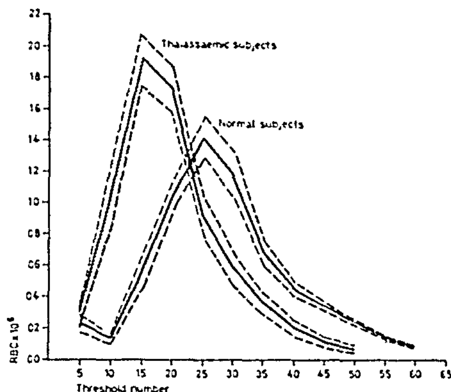


Fig 1

normal left peak In this way the volumetric distribution of thalassaemic blood may be clearly distinguished from that of normal samples (fig 1)

Further investigations have shown that the volumetric distribution of thalassaemic red cells may also be differentiated from samples of sideropenic subjects (fig. 2) In these patients the very high peak typical of thalassaemic blood is absent, while the initial platelet peak is usually, but not constantly, present. Schistocytosis is generally lower in sideropenic patients and is thus generally unable to completely mask the platelet peak and successive drop of the curve These findings are of considerable interest, as thalassaemic and sideropenic patients generally present very similar morphological pictures

On the basis of these findings, it may be concluded that the volumetric distribution of red blood cells presents a peculiar curve for thalassaemic blood. As the investigation technique used to obtain this curve is particularly easy and rapid, we suggest it as a suitable screening test for the thalassaemic trait.

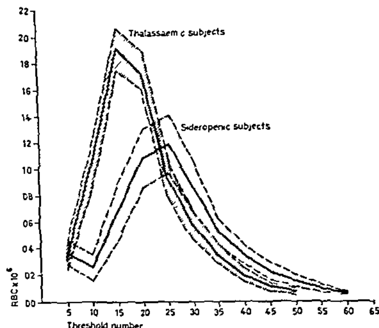


Fig 1 and 2 Volumetric distribution (Coulter B counter). Each dimensionless number on the abscissa indicates a specific instrument threshold setting, corresponding to pulse values, *the size of which is proportional to the size of the corresponding corpuscles from* which the pulses are registered Numbers on the ordinate indicate the number of pulses and thus of the cells present in the suspensions, counted and registered at each threshold setting ------ = Confidence limits 99%, ——— = mean

References

1 Brecher, G Jakobeck, E F, Schneiderman M A, Williams, G Z, and Schmidt, P J Size distribution of erythrocytes Ann. N Y Acad. Sci. 99 242 (1962)

2 Bull, B S *On the distribution of red cell volumes Blood 31 503–515 (1968)*

3 Cook, J S Size determination of human erythrocytes with an electronic counter J Lab clin Med. *70* 849–856 (1967)

4 Crane, K. and Waft, A. D Red cell volume distribution histograms for establishing *the normal range using the Coulter plotter model J* clin. Path *20* 913–915 (1967)

5 Dacie, J V and Lewis, S M Practical haematology, 4th ed. (Churchill, London 1970)

6 FRANK, M; ORTALI, V. e PLOLISI, G.: La valutazione numerica e dimensionale di corpuscoli sospesi in un mestruo conduttore. Rend. Ist. sup. Sanità 26. 700–726 (1963).
7 GUTMANN, J; HOFMANN, G., and RUHENSTROTH BAUER, .: Exact methods for measuring the volume distribution of erythrocytes on the basis of the Coulter principle. Bibl. haemat., vol. 24, pp. 21–22 (Karger, Basel 1966).
8 INGRAM, M.: Frequency distribution histograms and MCV changes of various fractions of erythrocyte populations. Bibl. haemat., vol. 24, pp. 37–41 (Karger, Basel 1966).
9 LE GO, R.; LE GO, A. et CROULLIERE, R.: Le spectre des volumes cellulaires érithrocytes leucocytes plaquettes. Analyse de la distribution avec un sélecteur d'amplitudes multicanaux. Nouv. Rev. franç. Hémat. *10* 709–726 (1970)
10 LUNETTA, M. e CALCAGNO, G · La curva di Price-Jones determinata con un contaglobuli elettronico in 50 soggetti normali. Boll. Soc. ital Biol. sper. *43* 173–177 (1967).
11 LUSHBAUGH, C. C. and LUSHBAUGH, D. B · Rapid electronic red blood cell sizing as an aid in clinical diagnosis. Sth med. J. *58* 1208–1212 (1965).
12 MATTERN, C. F. T; FREDERICK, S. B., and OLSON, B. J. Determination of number and size and particles by electrical gating: blood cells. J. appl. Physiol *10* 56–70 (1957).
13 UR, A. and LUSHBAUGH, C. C.: Some effects of electrical fields on red blood with remarks on electronic red cell sizing. Brit. J. Haemat. *15* 527–538 (1968).

Author's address: Dr G TORLONTANO, Via Niccolò Piccinni 32, *00199 Roma* (Italy)

Acta Haemat. 48 239–244 (1972)

Quantitative Study of the Effects of Cyclophosphamide on Peritoneal Cell Populations

K. N. Chin and G. Hudson

Department of Haematology, University of Sheffield, Sheffield

Abstract. A quantitative study of peritoneal cell populations has been carried out in mice at intervals of 6 h to 30 days after a single sublethal dose of cyclophosphamide. There was a severe depletion in the number of lymphoid cells, which was first detected at 12 h and reached a maximum at 6 days. Macrophages were evident in the removal and disposal of degenerating cells, and showed increased numbers at 3 days. Recovery was rather slow, significant differences in small lymphocyte counts being still present at 18 days, and in other lymphocytes and blast cells at even later intervals.

Key Words
Cyclophosphamide
Cytostatics
Mouse peritoneum
Peritoneal cells

In earlier investigations [1, 6, 7] the effects of a single sublethal dose of cyclophosphamide on the bone marrow, blood and Peyer's patches of guinea pigs and mice have been studied in an attempt to throw light on the differing reactions of different elements of the lymphomyeloid complex. In the present investigation, a quantitative method [11] has been used to extend these observations to the peritoneal cell populations of mice, this does not seem to have been studied before.

Material and Methods

Healthy male albino mice of a closely inbred strain were used. Each was aged about 80 days and weighed between 30 and 40 g.

200 animals were given a single injection via the tail vein under light ether anaesthesia, and 10 were studied without injection. 100 of the injected mice, the 'test' series, received cyclophosphamide (Endoxana®, Ward, Blenkinsopp & Co) in a dose of 300 mg/kg body weight in a fluid volume of 0.25–0.35 ml, while the other

100, the 'control' series, received a corresponding volume of sterile physiological saline. Corresponding test and control animals were studied concurrently. The general effect on the test animals was similar to that described earlier [6, 7] in that about 10% of the body weight was lost over the first 3 days when the animals were a little off-colour, but thereafter no ill effects were apparent. 10 test and 10 control animals were studied at each of the following intervals after injection: 6, 12 and 24 h, 3, 6, 9, 12, 18, 24 and 30 days.

The method for quantitative study of peritoneal cells was essentially the same as that used previously [11]. Immediately following death in an ether chamber, 4 ml of physiological saline was injected into the peritoneal cavity. The abdomen was gently massaged for 15 sec, after which the abdominal cavity was opened and as much fluid as possible removed; this was usually just less than 4 ml. The fluid was then centrifuged at 1,400 rpm for 5 min. The resulting cell suspension was re-suspended in about 1 ml of supernatant fluid and 0.2 ml of a mouse liver extract prepared by the method of FICHTELIUS [3]. The total volume was measured. Standard haemocytometric methods were used to count the number of nucleated cells per mm³ of this suspension and from this, the absolute numbers of cells originally collected could be estimated.

The suspension was again centrifuged and the cell pellet re-suspended in a few drops of commercial horse serum. Smears were prepared from this suspension, air-dried and stained with MacNeal's tetrachrome. In each preparation, all the cells were classified in two complete traverses across the centre of a smear at right angles to its long axis, not less than 400 cells being counted. The classification of cells was also similar to that used previously [11], viz. small lymphocytes, other lymphocytes, blast (or blastoid) cells, macrophages (including monocytes), mast cells, eosinophils, neutrophils and damaged forms. The cells termed blast (or blastoid) were usually large cells of 15 μm or more in diameter with a complete rim of basophilic cytoplasm surrounding a round and centrally placed nucleus. The cytoplasm occasionally showed a vacuole or a perinuclear pale area while the nucleus showed a moderately fine chromatin and one or more nucleoli; very occasionally, the nucleus had a bilobed form.

By relating the percentage figures so obtained to the total counts, the absolute number for each cell group was finally calculated.

Results

The average counts, ± standard deviations, for the main cell groups are recorded in table I, only highly significant differences between corresponding test and control values ($p<0.01$) being indicated.

In examining the smears of test animals in the early stages after injection, some evidence of cell degeneration in the form of pyknosis and fragmentation of nuclei was present. These appearances were particularly prominent at 12 and 24 h but were seen at all intervals till 12 days. The

Table 1 Average counts in millions ± standard deviations

		Total nucleated	Small lymphocytes	Other lymphocytes	Blast cells	Macrophages + monocytes	Mast cells	Eosinophils	Neutrophils	Damaged forms
0 h		7 27±2 35	0 54±0 33	0 96±0 49	1 61±0 75	[illegible]	[illegible]	[illegible]	0 02±0 03	1 91±0 86
6 h	T	6 20±1 66	0 38±0 36	0 79±0 39	0 98±0 57	[illegible]	[illegible]	[illegible]	0 03±0 03	[illegible]
	C	6 63±1 32	0 37±0 13	1 07±0 27	0 97±0 40	[illegible]	[illegible]	[illegible]	0 02±0 02	[illegible]
12 h	T	4 81±2 07	0 19±0 13[1]	0 33±0 22[1]	0 59±0 36	[illegible]	[illegible]	[illegible]	0 07±0 07	[illegible]
	C	6 13±2 49	0 51±0 27	1 36±0 64	1 32±0 84	[illegible]	[illegible]	[illegible]	0 02±0 02	[illegible]
24 h	T	4 60±1 11[1]	0 12±0 09[1]	0 22±0 14[1]	0 43±0 18[1]	[illegible]	[illegible]	[illegible]	0 19±0 24	[illegible]
	C	6 55±1 80	0 63±0 28	1 02±0 32	1 19±0 66	[illegible]	[illegible]	[illegible]	0 04±0 07	[illegible]
3 days	T	3 85±0 49[1]	0 10±0 13[1]	0 12±0 10[1]	0 30±0 23[1]	[illegible]	[illegible]	[illegible]	0 03±0 03	[illegible]
	C	6 64±1 25	0 64±0 37	1 21±0 38	1 51±0 69	[illegible]	[illegible]	[illegible]	0 03±0 03	[illegible]
6 days	T	2 71±0 58[1]	0 07±0 08[1]	0 11±0 09[1]	0 15±0 11[1]	[illegible]	[illegible]	[illegible]	0 02±0 04	[illegible]
	C	6 69±1 32	0 60±0 38	1 31±0 59	1 22±0 38	[illegible]	[illegible]	[illegible]	0 01±0 01	[illegible]
9 days	T	2 60±0 99[1]	0 14±0 14[1]	0 24±0 22[1]	0 13±0 07[1]	[illegible]	[illegible]	[illegible]	0 12±0 20	[illegible]
	C	7 74±2 85	0 96±0 52	2 13±1 15	1 33±0 63	[illegible]	[illegible]	[illegible]	0 03±0 05	[illegible]
12 days	T	3 50±1 27[1]	0 08±0 06	0 22±0 19[1]	0 29±0 18[1]	[illegible]	[illegible]	[illegible]	0 10±0 10	[illegible]
	C	6 92±2 50	0 75±0 75	1 29±0 55	1 12±0 46	[illegible]	[illegible]	[illegible]	0 01±0 01	[illegible]
18 days	T	4 93±1 76[1]	0 30±0 20[1]	0 49±0 32[1]	0 35±0 16[1]	[illegible]	[illegible]	[illegible]	0 12±0 11[1]	[illegible]
	C	8 13±1 84	1 00±0 54	1 68±0 58	1 41±0 60	[illegible]	[illegible]	[illegible]	0 02±0 03	[illegible]
24 days	T	4 79±1 08[1]	0 36±0 22	0 70±0 46[1]	0 56±0 35[1]	[illegible]	[illegible]	[illegible]	0 03±0 03	[illegible]
	C	7 89±2 87	0 59±0 38	1 51±0 71	1 44±0 50	[illegible]	[illegible]	[illegible]	0 01±0 02	[illegible]
30 days	T	4 77±2 01	0 37±0 29	0 64±0 41	0 29±0 22[1]	[illegible]	[illegible]	[illegible]	0 05±0 05	[illegible]
	C	6 27±2 44	0 55±0 43	1 19±0 56	1 07±0 66	[illegible]	[illegible]	[illegible]	0 02±0 03	[illegible]

T = Test, C = control
[1] Significance at 0 01 level of probability

cells affected appeared to be lymphocytes, blast cells and less frequently, eosinophils and neutrophils but normal looking cells of all types were seen at every interval. In 0 h and control smears macrophages containing recognisable cells were rarely seen, but in the early stages after cyclophosphamide, macrophages containing lymphocytes, mast cells, eosinophils or neutrophils were readily identified. At 3 days, some of the macrophages appeared to be about twice the normal size, and showed prominent surface projections and abundant vacuolation. Binucleated macrophages were occasionally seen. No abnormal appearances were seen in test smears at or after 18 days. Mitoses were only occasionally seen in 0 h and control smears, rare examples were still encountered in smears taken during the early stages after cyclophosphamide.

Discussion

Although it is open to question whether the absolute counts of cells obtained by this method are an accurate representation of the actual number of cells present *in vivo*, differences in cell counts between corresponding test and control animals may be interpreted as reflecting changes in peritoneal cell populations. Caution must, however, be exercised because of concomitant changes in the numbers of unidentified damaged forms.

The most striking finding was the severe depletion of lymphoid cell numbers following the administration of cyclophosphamide. Table 1 shows that there were highly significant differences in the numbers of both small and other lymphocytes as early as 12 h and that by the 6th day, the average values were only about 10% of those of the corresponding controls. The changes in blast cells were very similar. The depletion in the total numbers of nucleated cells to a large extent reflected these changes in lymphocytes and blast cells.

The apparent sensitivity of the lymphoid population to cyclophosphamide is parallel to that already observed in the Peyer's patch [1] and bone marrow [5–7]. Bearing in mind that amongst the complex actions of alkylating agents [13] effects on DNA synthesis appear to be very important, one might expect particular effects on actively dividing cells and secondarily on cell populations derived from them. The striking depletion of lymphocyte numbers would therefore seem consistent with evidence [12] that peritoneal lymphocytes have the DNA labelling features of a rapidly proliferating population. They seem to be short lived and derived via the

blood stream from precursors in the bone marrow Long lived lymphocytes, on the other hand, are thought to be resistant to cyclophosphamide [9] Radiation chimera studies [4] have indicated that all peritoneal cell types come ultimately from haemopoietic tissue

In contrast to the lymphocytes and blast cells, the macrophages showed no evidence of depletion in numbers, on day 3, significantly higher counts were found The increased prominence of these cells following cyclophosphamide administration corresponded with that shown by macrophages of the Peyer s patch and bone marrow in similar circumstances [1, 7] The appearance of many laden macrophages in the early stages after cyclophosphamide would indicate their importance in the rapid removal of degenerating cells, cell debris, etc

In view of the smaller numbers of granulocytes present, firm conclusions concerning changes in these cells would not seem justified However, the apparent depletion of the eosinophil population at 24 h would be consistent with a similar depletion of blood eosinophils noted at this and subsequent intervals in the guinea pig [6, 7]

Table I also indicates that there was a depression of mast cell numbers which was first detected at day 6 and was still present at day 30 The fact that degranulation of mast cells and basophils can occur readily as a preparation artifact must be borne in mind [8], but it is of note that extensive damage to mast cells has been found following the use of nitrogen mustard [10]

Recovery of the peritoneal cell population from the effects of a sublethal dose of cyclophosphamide would seem from table I to have been rather slow, the total cell count still being low during the 4th week. Although the lowest counts of lymphoid cells were obtained on day 6, counts of small lymphocytes were still significantly below the control values on day 18, while the counts of other lymphocytes and blast cells were still depressed on days 24 and 30, respectively In the earlier observations in guinea pigs [5], the recovery of the small lymphocytes of the bone marrow followed a very similar time scale to that seen in peritoneal small lymphocytes In view of the evidence discussed above that peritoneal cells have their origin in the bone marrow, one is tempted to suggest that marrow transitional cells [14] which show an early recovery following cyclophosphamide administration [6] may be functioning as stem cells here It is significant that marrow stem cells, as studied by colony formation, have also recently been shown to recover early after cyclophosphamide [2]

Acknowledgement: We thank Mrs. Helen Pilling for secretarial help with the manuscript.

References

1 Chen, K. N. and Hudson, G.: Effects of cyclophosphamide on the ultrastructure of Peyer's patches. Brit. J. exp. Path. *51:* 536 (1970).

2 Dewys, W. D.; Goldin, A., and Mantel, N.: Hematopoietic recovery after large doses of cyclophosphamide: correlation of proliferative state with sensitivity. Cancer Res. *30:* 1692 (1970).

3 Fichtelius, K. E.: A factor in the liver scattering aggregates of lymphoid cells in artificially prepared suspensions. Acta path. microbiol. scand. *62:* 47 (1964).

4 Goodman, J. W.: On the origin of peritoneal fluid cells. Blood *23:* 18 (1964).

5 Host, H.: Regeneration of bone marrow cells in rats following cyclophosphamide or total body irradiation. Acta radiol., Stockh. *4:* 337 (1966).

6 Kaul, M. and Hudson, G.: Recovery of haemopoiesis after cyclophosphamide. Acta haemat., Basel *41:* 170 (1967).

7 Kaul, M. and Hudson, G.: Cell degeneration pattern in bone marrow and blood of guinea pigs following administration of cyclophosphamide. Acta haemat., Basel *42:* 42 (1969).

8 Michels, N. A.: The mast cells; in Downey Handbook of hematology, p. 231 (Hoeber, New York 1938).

9 Miller, J. J. and Cole, L. J.: Resistance of long lived lymphocytes and plasma cells in rat lymph nodes to treatment with prednisone, cyclophosphamide, 6-mercaptopurine and actinomycin D. J. exp. Med. *126:* 109 (1967).

10 Schoch, L. P. and Glick, D.: The effect of cold stress, ACTH, cortisone, pyrogen and nitrogen mustard on tissue mast cells in the skin and subcutaneous tissues of the rat. J. invest. Derm. *20:* 119 (1953).

11 Sherman, J.; Hunt, C.; Yoffey, J. M., and Hudson, G.: Observations on peritoneal fluid cells in mice. J. Anat., Lond. *102:* 140 (1968).

12 Volkman, A.: The origin and turnover of mononuclear cells in peritoneal exudates of rats. J. exp. Med. *124:* 241 (1966).

13 Wheeler, G. P.: Some biochemical effects of alkylating agents. Fed. Proc. *26:* 885 (1967).

14 Yoffey, J. M. and Courtice, F. C.: Lymphatics, lymph and the lymphomyeloid complex (Academic Press, London 1970).

Authors' address: Dr. K. N. Chen and Dr. G. Hudson, Faculty of Medicine, University of Sheffield, Sheffield 10 (England)

Acta Haemat. 48 245–250 (1972)

Failure of Subcutaneous Heparin Treatment in a Case of Disseminated Intravascular Coagulation

W Stevens, G Baele and G Verdonk

Department of Internal Medicine, State University of Ghent, Ghent

Abstract In a case of bronchogenic carcinoma with metastatic involvement of various sites, the typical clinical and laboratory abnormalities of disseminated intravascular coagulation were present Intravenous heparin treatment corrected the hemostatic parameters, while in contrast, subcutaneously administered heparin was completely ineffective

Key Words
Consumption coagulopathy
Fibrinogen degradation
Heparin
Intravascular coagulation

Disseminated intravascular coagulation (DIC) occurs in a wide variety of diseases Malignant tumors are occasionally complicated by a consumption coagulopathy [1, 4, 5, 11, 15] The mechanism of the DIC in these instances has not been fully elucidated Release of thromboplastins from the carcinoma, tissue invasion with damage to endothelium and microembolization are thought to be responsible for the initiation and continuation of the coagulation process [3] The effectiveness of intravenous heparin treatment is generally admitted [3, 8, 14] In many instances, however, it is impossible to continue the intravenous route of administration for a long time As no treatment of the underlying disease is effective in most cases of widespread carcinomatosis, the heparin administration must be continued and subcutaneously administered heparin would be the logical form of treatment

We want to report a case of DIC, due to bronchogenic carcinoma, in which intravenous heparin treatment initially improved hemostatic parameters and subcutaneously administered heparin subsequently failed to maintain this amelioration Cyclophosphamide, in single intravenous doses of 1 g, could not influence distinctly the course, neither in a positive nor in a negative way

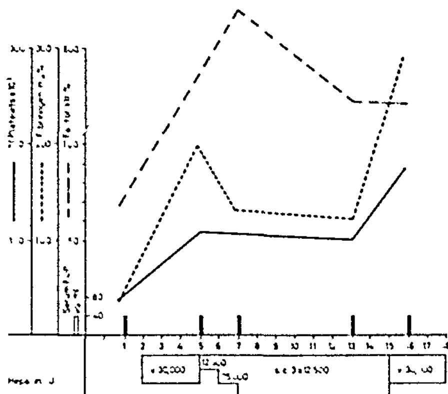


Fig. 1. Influence of intravenous heparin administration

Case Report

A 64 year old woman was admitted to the hospital with general complaints of anorexia, fatigue and weight loss. Seven years prior to admission a right lower lobectomy *for bronchogenic carcinoma was performed. For about 4 months she had* noticed multiple ecchymoses appearing spontaneously or after minor trauma. No other bleeding localizations were present.

On physical examination multiple ecchymoses were noted on the extremities. The edge of the liver was felt 6 cm below the right costal margin and multiple liver nodules were palpated. Spontaneous pain and pressure tenderness were present on the cervical and lumbar portion of the spine. Routine laboratory findings were normal except for slightly raised transaminases and alkaline phosphatases. Peripheral blood and bone marrow examination were normal. A liver scan permitted to state a diffusely enlarged liver with multiple irregular defect pictures. A chest roentgenogram revealed a normal status after lobectomy. Spine X rays showed a lytic lesion of C 7.

Coagulation results at admission. Platelets 37 000/mm³. Quick time 23.6 sec or 31%. Activated partial thromboplastin time (CBT) longer than 3 min. Fibrinogen

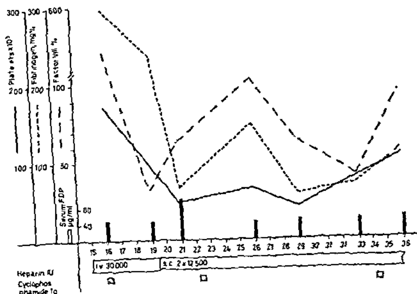


Fig 2 Influence of intravenous cyclophosphamide administration

In o... to correct the consumption coagulopathy, we ...
first da...
the intr...
(hepari...
sults of the assays of F VIII, fibrinogen, serum FDP and platelet enumeration are
s... figure 1. On the 15th day of treatment ecchymoses reoccurred and a con-
t...

From the ...
ment, we again administered 1 g cyclophosphamide intravenously on day 22 and 34

Figure 3 shows the results of the coagulation parameters used during the prolonged subcutaneous heparin treatment. Subcutaneous heparin was injected in a single morning dose of 25,000 IU starting from the 41st day. Some weeks after discharge the patient died in cardiovascular collaps.

Methods

Platelet enumeration was performed with a Coulter counter. The one stage prothrombin time (PT) was done using human brain thromboplastin according to

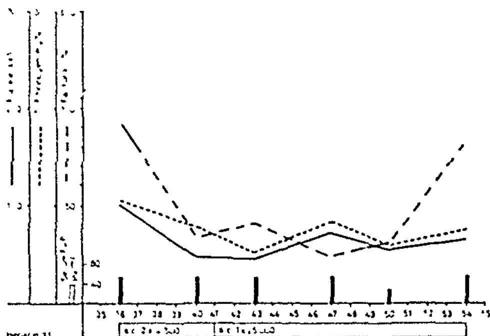


Fig. 3. Influence of subcutaneous heparin administration.

the method of QUICK *et al* [10]. The activated partial thromboplastin time (cephalin bentonite time, CBT) was performed according to Dr POTTER and OTTEN VAN DEN BERGHE [2]. Fibrinogen was determined using the method of VERMYLEN *et al* [13]. Serum FDP were determined by a hemagglutination inhibition immunoassay [9] with the Burroughs Wellcome kit. Factor VIII one stage assay was performed with the method proposed by SOULIER and LARRIEU [12].

Discussion

To our present knowledge the effectiveness of subcutaneous heparin administration in chronic cases of DIC has not been extensively studied in the literature. It is generally accepted that intravenous administration of heparin can correct the consumption coagulopathy. Disseminated intravascular coagulation has been called an intermediary mechanism of disease [6]. Heparin treatment may be used to prevent consumption of coagulation factors and platelets until the underlying disease has been eradicated and the consumptive process stopped. However, in most cases of carcinomatosis with DIC, no effective treatment of the primary disease is

feasible and heparin treatment *is only able to prolong life* Home care with continuous intravenous heparin administration is highly impossible Subcutaneous administration of heparin would be an attractive alternative as vitamin K antagonists are usually ineffective [7, 8] In our patient we saw a marked *rise of fibrinogen*, platelets and factor VIII during both intravenous heparin treatment periods, despite continuously elevated serum FDP levels

In contrast, subcutaneous heparin treatment was very disappointing because *all the parameters used fell to their* low initial levels. Cyclophos-[illegible] was given in an attempt to influence the need to heparin No

[illegible]

gravation of the consumption coagulopathy was noted but after the initial cyclophosphamide injection the hemostatic parameters showed a trend to rise

In conclusion, subcutaneous heparin administration failed to be an effective treatment in the presented case of chronic DIC due to widespread metastases of bronchogenic carcinoma It seems worthwhile to evaluate further the indications, if any, of subcutaneous heparin in the treatment of chronic DIC.

References

1 CYR, D P and METHA, V B Disseminated intravascular clotting. *Med Clin N Amer 53* 301 (1969)

2 POTTER, E DE et OFFNER VAN DEN BERGHE, C. L emploi d un nouveau test de controle de la coagulation intrinseque Acta haemat., Basel *28* 370 (1962)

3 HOROWITZ, H I Treatment of defibrination syndromes (other than pregnancy) and fibrinolytic disorders in *Treatment of hemorrhagic disorders* (Harper & Row New York 1968)

4 KARPATKIN M Diagnosis and management of d sseminated intravascular coagulation Pediat. Clin. N Amer *18* 23 (1971)

5 KWAAN H C Disseminated intravascular coagulation Med Clin N Amer *56* 177 (1972)

6 MC KAY D G Disseminated intravascular coagulation an intermediary mechanism of d sease (Harper & Row New York 1965)

7 MERSKEY C JOHNSON A J KLEINER, G J., and WOHL, H The defibrination syndrome clinical features and laboratory diagnosis. Brit. J Haemat *13* 4 (1967)

8 MERSKEY C JOHNSON A J PERT J H., and WOHL, H Pathogenesis of *fibrinolysis* in defibrination syndrome effect of heparin administration. Blood *24* 701 (1964)

9 MERSKEY, C., LALEZARI, P., and JOHNSON, A. J.: A rapid, simple, sensitive method for measuring fibrinolytic split products in human serum. Proc. Soc. exp. Biol. Med. *131:* 871 (1969).
10 QUICK, A. J., STANLEY-BROWN, M., and BANCROFT, F. W.: A study of the coagulation defect in hemophilia and in jaundice. Amer. J. med. Sci. *190:* 501 (1935).
11 RODRIGUEZ-ERDMANN, F.: Bleeding due to increased intravascular coagulation. New Engl. J. Med. *273:* 1370 (1965).
12 SOULIER, J. P. et LARRIEU, M. J.: Nouvelle méthode de diagnostic de l'hémophilie. Dosage des facteurs antihémophiliques A et B. Sang *24:* 3 (1953).
13 VERMYLEN, C., VERSTRAETE, M., and VREKER, R. A. DE: A simple method for the instantaneous quantitation of fibrinogen: the FPT test. Acta clin. belg. *18:* 200 (1963).
14 VERSTRAETE, M., AMERY, A., and VERMYLEN, C.: Diagnostic hints for intravascular coagulation and its treatment with heparin. Acta clin. belg. *18:* 450 (1963).
15 VERSTRAETE, M., VERMYLEN, C., VERMYLEN, J., and VAN DEN BROUCKE, J.: Excessive consumption of blood coagulation components as cause of hemorrhagic diathesis. Amer. J. Med. *38:* 899 (1965).

Authors' address: Dr. W. STEVENS, Dr. G. BAELE and Prof. Dr. G. VERDONK, Akademisch Ziekenhuis, De Pintelaan 135, *B 9000 Gent* (Belgium)

Acta Haemat. 48 251–255 (1972)

Localisations mammaires d'une leucémie aigue lymphoblastique

G HŒRNI SIMON, B HŒRNI, J CHAUVERGNE et M DURAND

Fondation Bergonie (Directeur Prof C LAGARDE) Bordeaux

Abstract A case of acute lymphoblastic leukaemia with massive lesions localized in the breast tissue is reported. This unusual feature its etiology and the possibilities for treatment of these tumours are discussed

Key Words
Acute lymphatic leukaemia
Breast involvement in leukaemia
Mammography

L'atteinte macroscopique des seins au cours d'une leucemie aigue lymphoblastique est exceptionnelle A l'occasion d un cas que nous avons observe, nous voulons souligner quelques caractères interessants de cette localisation

Observation

Mme M T Mon (FB 71 1595) 21 ans est hospitalisee à la Fondation Bergonié le 12 aout 1971 pour le bilan et le traitement d une leucemie aigue lymphoblastique (LAL) C est l hémogramme pratique à la suite d une hemorragie secondaire a l avortement spontané d un fœtus de 5,5 mois, qui a permis de porter ce diagnostic. Dans ses antécedents on relève une syphilis decouverte lors d un examen prénuptial 17 mois plus tot, traitée et guerie Elle est mere d un enfant de 6 mois

A son entree elle est apyretique mais se plaint d une anorexie et d une asthenie modérees Outre les signes cliniques habituels de LAL (polyadénopathie superficielle hepatomegalie et splénomégalie), on note une importante hypertrophie mammaire bilaterale les deux seins sont globuleux, tendus, douloureux, de consistance homogene (fig 1) Le reste de l examen clinique est negatif L hemogramme avec 193 000 leucocytes/mm³ (dont 90% de blastes) et le medullogramme confirment le diagnostic de LAL, de type macrolymphoblastique

La radiographie pulmonaire est normale la lymphographie met en evidence une atteinte diffuse et symétrique des ganglions iliolombaires. La mammographie objective deux glandes très volumineuses avec un tissu cellulaire sous-cutane infiltre et



Fig. 1. Hypertrophie mammaire bilatérale

œdémateux, une peau épaissie traduisant une affection en phase aiguë (fig. 2). La ponction à l'aiguille fine des deux glandes confirme leur envahissement par le processus leucémique (fig. 3). Par ailleurs, le liquide céphalorachidien est acellulaire et, sur le plan biochimique, on ne remarque qu'une hyperuricémie à 173 mg/l.

Après 48 h d'une réanimation hématologique associant transfusions, cure de diurèse, urate-oxydase et allopurinol, on institue un traitement général comportant vincristine, daunorubicine et prednisone, que l'on complète 15 jours plus tard par une cobalthérapie des deux glandes mammaires : une dose de 1400 R/T en 2 semaines est jugée suffisante en raison du retour rapide à la normale du volume et de la consistance des deux seins.

Après une brève période d'amélioration de un mois avec chute leucocytaire à 20 000/mm³, l'état clinique et hématologique s'aggrave de nouveau. On commence alors un traitement par L-asparaginase en même temps qu'une cobalthérapie splénique, à visée réductrice, compte tenu de la radiosensibilité constatée antérieurement sur les localisations mammaires. Or la splénomégalie s'avère pratiquement insensible à une dose de [illegible] rads et le développement d'une septicémie à staphylocoques avec localisations cutanées et musculaires fait suspecter l'existence dans la rate d'un réservoir staphylococcique. On décide donc une splénectomie réalisée le 26 octobre qui ne permet de constater qu'un envahissement lymphoblastique massif. La malade décède le 27 octobre 1971 dans un tableau de septicémie à colibacille avec état de choc.



Fig 2 Mammographie droite Profil

La nécropsie a permis de retrouver des infiltrats leucoblastiques dans le foie, les deux surrénales, un ganglion médiastinal et la moelle osseuse, les deux seins sont indemnes d'atteinte spécifique et présentent une atrophie avec dysplasie fibreuse diffuse.

Commentaires

A l'occasion de cette observation, nous soulignerons seulement plusieurs points intéressants posés par les localisations mammaires macroscopiques des LAL.

Si l'envahissement microscopique des seins s'observe fréquemment à la phase ultime de l'évolution [2], au contraire leur atteinte précoce et macroscopique est exceptionnelle puisque KENNEDY *et al* [1] n'ont pu recenser que 6 cas antérieurs au 7e, personnel, qu'ils rapportent. Ces atteintes s'observent le plus souvent chez des femmes de 20 à 40 ans et, en général, lors d'une rechute de l'évolution leucémique. Qu'elles soient uni ou bilatérales, leur aspect clinique n'a aucun caractère particulier et

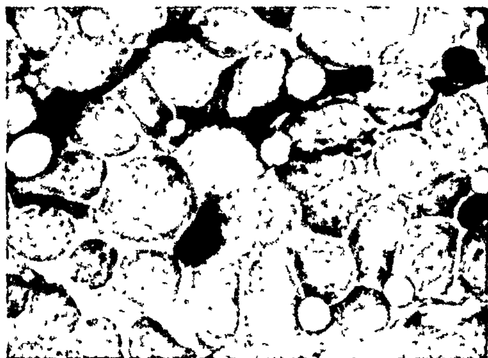


Fig. 3 Cytologie mammaire May Grunwald Giemsa × 1200

évoque seulement un cancer à croissance rapide de type mastite aiguë carcinomateuse. La mammographie, exceptionnellement faite, apporte des éléments du même ordre : infiltration dense et diffuse avec œdème cutané.

La pathogénie de cette localisation mammaire n'est pas précisée mais on peut remarquer qu'il existe, dans notre observation comme dans plusieurs autres précédemment publiées, une relation avec un épisode de la vie génitale et en particulier avec un avortement. Il est possible qu'à cette occasion le parenchyme glandulaire des seins subisse des modifications métaboliques qui en font un terrain propice à la colonisation par les leucoblastes.

Enfin, contrairement à l'opinion exprimée par SELLEI *et al.* [3], il semble que cette localisation tumorale n'aggrave pas l'évolution. Elle constitue simplement l'indication d'une irradiation limitée qui, dans notre observation, a permis, en association avec la chimiothérapie, d'obtenir une stérilisation des glandes mammaires. Si KENNEDY *et al.* [1] ont pu obtenir le même résultat avec la seule chimiothérapie, il semble que

l'irradiation à doses faibles constitue un adjuvant intéressant par sa commodité et la rapidité de ses effets, son utilisation, en réalisant une réduction tumorale appréciable, s'insérant d'ailleurs dans l'orientation actuelle du traitement des LAL.

Résumé

L'observation de localisations mammaires massives de LAL est rapportée. La rareté, les conditions cliniques et l'effet thérapeutique de ces manifestations tumorales sont soulignées.

Bibliographie

1 KENNEDY, B. J., BORNSTEIN, R., BRUNNING, R. D., and DUNN, D. Breast involvement in acute lymphatic leukemia. Daunorubicine-induced remission, pneumocystis carinii pneumonia. Cancer, [illegible] (19[illegible]).
2 SANDISON, A. T. Metastatic tumours in the breast. Brit. J. Surg. [illegible] (19[illegible]).
3 [illegible], G., [illegible], J., [illegible], C. et [illegible], L. Localisations mammaires des hémopathies malignes. I. Clinique. II. Histologique. Pathogénie. Aspects radiologiques. Presse méd. 70: 2771-2774 et 2821-2822 (1962).

Adresse des auteurs: Dr G. HŒRNI-SIMON, Dr B. HŒRNI, Dr J. CHAUVERGNE et Dr M. DURAND, Fondation Bergonié, 180, rue de Saint-Genès, F-33076 Bordeaux (France)

D. HARMS: Postmortale Fibrinolyse beim Menschen. Veröffentlichungen aus der morphologischen Pathologie, Heft 87. Herausgegeben von W. GIESE, W. BÜNGELER, G. SEIFERT und G. PETERS. Fischer, Stuttgart 1971. XII, 74 pp., 18 fig., DM 35.–.

Die Monographie gibt die Resultate einer genau durchgeführten experimentellen Arbeit über Häufigkeit, Stärkegrad und Art der postmortalen Fibrinolyse wieder. Es wird anhand von Untersuchungen über das Schicksal der Thrombozyten im Leichenblut geschlossen, dass das postmortale Verschwinden des Fibrinogens niemals einer primären Fibrinogenolyse zuzuschreiben ist, sondern das Resultat einer intravasalen Gerinnung mit sekundärer Fibrinolyse ist. Obschon es in einer solchen Studie unmöglich ist, alle Parameter zu berücksichtigen, ist es schade, dass der Gerinnungsablauf, mindestens in einigen Fällen, nie untersucht wurde. Es wäre sehr instruktiv, während der ersten Phase quantitative Angaben über Fibrinogen, Prothrombin und noch andere Gerinnungsfaktoren zu sammeln. Die Fortschritte der Fibrinolyse hätte man mit dem Abfall der Plasminogenaktivität und der Zunahme und immunologischen Charakterisierung der Fibrin- und Fibrinogenspaltprodukte dokumentieren können. Auch für diese Studie wäre es vorteilhaft gewesen, quantitative Methoden für die Bestimmung des Fibrinogens und der Plasminogenaktivität zu verwenden. Es hätte die Interpretation einiger Resultate erleichtert. Dennoch wird klar bewiesen, dass die Fibrinolyse nach einer Latenzperiode aktiviert wird und dass diese Fibrinolyse aktiv genug ist, um Mikrothromben, eventuell auch Makrothromben, nach intravasaler Gerinnung *intra vitam* aufzulösen. Dieser Punkt ist für den autoptischen Nachweis einer intravasalen Gerinnung besonders wichtig.

Diese Monographie ist eine wertvolle Quelle von präzisen Informationen, für den an dieser spezifischen Fragestellung interessierten Leser. P. DUCKERT, *Basel*

Acta Haemat. 48: 257–268 (1972)

Inactive Factor VIII in Hemophilia A and Willebrand's disease

A Study of 117 Cases[1]

K. Lechner

Central Coagulation Laboratory (Head: Doz. Dr. K. Lechner),
First Department of Medicine (Head: Prof. Dr. E. Deutsch),
University of Vienna, Vienna

Key Words
Blood coagulation
Factor VIII antibody neutralization
Hemophilia
Von Willebrand's disease

Abstract. The inhibitor neutralizing capacity of the plasmas of 101 patients with hemophilia A and 16 patients with von Willebrand's disease was determined, using a natural antibody against factor VIII. In 48 patients with severe hemophilia A, no inhibitor-neutralizing material was detectable. The plasmas of 47 patients with moderate or mild hemophilia A contained inhibitor neutralizing material in amounts equivalent to the biological activity. In 6 patients with hemophilia A (factor VIII activity ranging from 3.2–26%), from 5 hundreds more inhibitor neutralizing material was found as expected from the biological activity, indicating the presence of inactive, probably structurally abnormal factor VIII. Inactive factor VIII was not found in plasmas of patients with von Willebrand's disease.

The antibody neutralization technique has become a new important tool for the study of congenital and acquired clotting factor deficiencies. Using this technique, subgroups of congenital coagulation disturbances could be defined according to the presence or lack of an 'inactive' coagulation protein, which is thought to be present when the immunological assay gives higher values than the biological method for the determination of a clotting factor.

In this study, the results of an immunological and a biological assay of factor VIII were compared in 101 patients with hemophilia A and 16 patients with von Willebrand's disease. For the immunological determina-

[1] Supported by a grant of the 'Österreichischer Fonds zur Förderung der wissenschaftlichen Forschung' No. 903.

in this study. It is given either by the amount of neutralized inhibitor (= difference between the mean value of the residual inhibitor of all buffer assays and the residual inhibitor after preincubation of the inhibitor with the plasma sample to be tested) or as percent of normal (this value is derived from the regression line shown in fig. 2 and 3).

Biologically active factor VIII: Factor VIII activity of plasma measured with the one-stage method as described in methods.

Inactive factor VIII (non functional factor VIII): Difference between immunoreactive and biologically active factor VIII (see also discussion).

Results

Reproducibility of the test system: To check the reliability of the inhibitor-neutralizing test, tests were performed each day with buffer, standard factor-VIII-deficient plasma, standard normal plasma and, in 18 occasions, with plasma of a patient with hemophilia A⁺. The results are shown in table I. As can be seen, the standard error of the method is 10% or less.

Inhibitor neutralization by plasmas of patients with severe hemophilia A. Plasmas of 48 patients with severe hemophilia A were tested for their inhibitor-neutralizing capacity. The results are shown in figure 1. The residual inhibitor activities ranged from 1.56 to 2.45 U/ml (mean 2.02 U/ml, standard deviation 0.19).

In the same figure, the individual values using standard factor-VIII-deficient plasma are shown. It is clearly seen from figure 1 and table I that the mean and the standard variation are quite similar, using standard factor-VIII deficient plasma, buffer or individual plasmas of patients with severe hemophilia A. Therefore, the different results obtained with different patient plasmas may be ascribed to variation of the method rather than to different amounts of immunoreactive factor VIII. This would mean that none of these plasmas contained significant amounts of inhibitor neutralizing material.

Inhibitor neutralization by plasmas of hemophilic patients with factor >1%. Plasmas of 53 patients with factor-VIII deficiency of various degrees were tested for their inhibitor neutralizing capacity and the results were compared to the value expected from the biological activity. The expected values are represented by a regression line obtained by plotting the residual inhibitor (U/ml) against factor-VIII activity (corresponding dilutions of normal plasma up to 1:5). The results are shown in figure 2. The

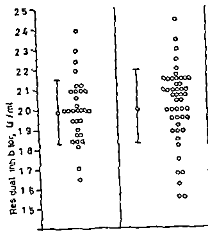


Fig 1 Left column Residual factor VIII inhibitor after preincubation of the inhibitor with standard factor VIII-deficient plasma (n=32) Right column Residual factor VIII inhibitor after preincubation of the inhibitor with plasmas of patients with severe hemophilia A (n=48)

values obtained with 33 plasmas were within the 95 percent limits of confidence of the regression line These plasmas may be considered to contain an amount of inhibitor neutralizing material equivalent to the biological activity The results obtained with 14 plasmas were between the 95- and 99 percent limits of confidence of the regression line For these plasmas, it cannot be decided whether their inhibitor neutralizing capacity corresponds to the value expected from the biological activity or whether an excess of inhibitor-neutralizing material is present

For the plasmas of 6 patients, however, it seems to be almost certain that they contain an excess of inhibitor neutralizing material, as these values were definitely outside the 99 percent limits The data of these patients are given in table II They belong to 5 different families The patients St E and St J are brothers The biological factor-VIII activity ranged from 3 2 to 26% The amount of immunoreactive factor VIII varied greatly from patient to patient Even in the brothers St E and St J, the amount of immunoreactive factor VIII was not identical, the difference being statistically significant ($p<0\ 01$) If compared to the inhibitor-neutralizing capacity of normal plasma, St. J had a significantly ($p<0\ 05$) higher amount of immunoreactive factor than normal plasma, the values

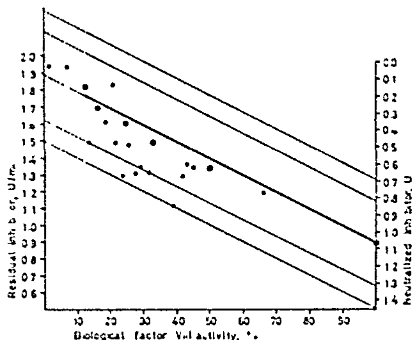


Fig. 3. Inhibitor neutralization by plasmas of patients with von Willebrand's disease. ● Residual inhibitor (U/ml) after preincubation of the inhibitor with dilutions of normal plasma (each point is the mean of at least 7 assays). ○ Residual inhibitor after preincubation of the plasmas of patients with von Willebrand's disease. ●——● Regression line (with confidential limits for 95 and 99%) calculated from the values obtained with dilutions of normal plasma.

first described independently by DENSON *et al* [4] and HOYER and BRECKENRIDGE [9]. Subsequently, similar findings were reported by FEINSTEIN *et al* [7] and LARRIEU and MEYER [11]. According to a suggestion of DENSON *et al* [4], this form of the disease, in which factor VIII is detected by the immunological method in higher amounts as expected from the biological activity, is called hemophilia A⁺, whereas the more frequent type, in which no inactive factor VIII can be demonstrated, is called hemophilia A⁻.

As the number of patients with hemophilia A⁺, so far described, is still limited, it seemed to be worthwhile to study the frequency of this variant of hemophilia A in a larger patient group, including patients with all degrees of severity of this disease. Further, an attempt was made to obtain quantitative data on the amount of immunoreactive and inactive factor VIII in those patients who were found to have hemophilia A⁺.

The method which was used for the determination of immunoreactive factor VIII was similar to that described by DENSON *et al.* [4] The only differences were shorter incubation times in our inhibitor assay (30 min instead of 120 min) and the use of a one stage method for the assay of factor VIII (instead of the two-stage method)

An important precondition for the performance of this study was to demonstrate that the test system was sufficiently stable over the whole period of the experiment and the results were comparable from day to day To check the reliability of the test system, each day several standard samples were run simultaneously with the plasma samples to be tested Considering that the method is rather complicated and involves many stepts, the reproducibility of the results may be regarded as satisfactory On the other hand, the results were not so constant that the method can be expected to detect smaller differences in the amount of inactive factor VIII

Another precondition for the detection of inactive factor VIII was to establish a normal relationship between biological activity and inhibitor-neutralizing capacity Assuming that normal plasma with a biological activity of 100% contains also 100% immunoreactive factor VIII, various dilutions of normal plasma representing various factor VIII activities were tested for their inhibitor neutralizing capacity It was found that a linear relationship exists between biological activity and inhibitor neutralizing capacity The limits of confidence for 95 and 99% were calculated regarding the variation of the single points, from which the regression line was calculated. A plasma was considered to have inactive factor VIII *if the value of immunoreactive factor VIII* was outside the 99 percent belts In all cases in which the value was within the 95 percent limits, it was assumed that the inhibitor neutralizing capacity corresponded to the value expected from the measured biological activity

Using these parameters, the plasmas of 6 patients out of 101 patients with hemophilia A were found to contain significant amounts of inactive factor VIII All these patients had a biological factor-VIII activity of more than 1%. In none of the patients with severe hemophilia A (factor VIII less than 1%) could inactive factor VII be detected with certainty Thus, in our material the frequency of hemophilia A$^+$, related to all tested patients with hemophilia A, was 5 95% This finding is in good agreement with the incidence reported by FEINSTEIN *et al* [7] (3 8%) and DENSON *et al* [4] (8.3%) LARRIEU and MEYER [11] and HOYER and BRECKENRIDGE [9] found an higher incidence (10 3% and 17.54%) Such a comparison is, however, problematic, for two reasons First, the proportion of severely

5 Denson, K. W. E., Biggs, R., and Mannucci, P. M.: An investigation of three patients with Christmas disease due to an abnormal type of factor IX. J. clin. Path. *21:* 160 (1968).

6 Lanti, P. and Sawyer, R. J.: Anticoagulant specificity and physiologically inactive beta thromboplastin. Nature, Lond. *177:* 1233 (1956).

7 Feinstein, D.; Chong, M. N. Y.; Kasper, C. K., and Rapaport, S. I.: Hemophilia A: polymorphism detectable by a factor VIII antibody. Science *163:* 1071 (1969).

8 Hellem, A. J.: Platelet adhesiveness in von Willebrand's disease. A study with a new modification of the glass bead method. Scand. J. Haemat. *7:* 374 (1970).

9 Hoyer, L. W. and Breckenridge, R. T.: Immunologic studies of antihemophilic factor (AHF, factor VIII): cross-reacting material in a genetic variant of hemophilia A. Blood *32:* 962 (1968).

10 Josso, F.; Beguin, S. et Weilland, C.: Variantes génétiques de l'hémophilie B. Thromb. Diath. haemorrh. (suppl.) *63:* 17 (1971).

11 Larrieu, M. J. and Meyer, D.: Heterogeneity of factor VIII and factor IX variants. Thromb. Diath. haemorrh. (suppl.) *63:* 11 (1971).

12 Lechner, K.: Genetische Varianten der Hämophilie B (im Druck).

13 Niessner, H.: Messung der Plättchenadhäsivität mit einer modifizierten Form der Hellem II Methode unter besonderer Berücksichtigung des v. Willebrand-Jürgens-Syndroms. Thromb. Diath. haemorrh. *27:* 434 (1972).

14 Pfanzagl, J.: Allgemeine Methodenlehre der Statistik, vol. 2 (Gruyter, Berlin 1966).

15 Roberts, H. R.; Grizzle, J. E.; McLester, W. D., and Penick, G. D.: Genetic variants of hemophilia B: detection by means of a specific PTC inhibitor. J. clin. Invest. *47:* 360 (1968).

16 Zimmerman, T. S.; Ratnoff, O. D., and Powell, A. E.: Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against antihemophilic factor. J. clin. Invest. *50:* 244 (1971).

Author's address: Dr. K. Lechner, I. Medizinische Universitätsklinik, Spitalgasse 23, A-1097 Wien (Austria)

Acta Haemat. 48: 269–277 (1972)

Pentose Phosphate Pathway of Erythrocytes in Uremia

T. MARKKANEN[1], O. PELTOLA, J. FORSSTRÖM and P. HIMANEN

Departments of Medicine and Medical Microbiology, University of Turku, Turku

Abstract. The transketolase activity (TKA) of red cells was determined in 286 controls and 50 patients with uremia. The TKA of the latter tended to exceed that of the controls, but statistically the difference was not significant. In the uremic patients, the TKA showed a positive correlation with serum creatinine, urea and potassium levels, and a negative correlation with the patients' hemoglobin and hematocrit. A 10-hour dialysis did not affect the TKA of the red cells in uremic patients, nor did uremic plasma inhibit or activate the TKA *in vitro*.

Key Words
Erythrocyte metabolism
Pentose phosphate pathway
Transferases
Transketolase
Uremia

A preliminary observation we have reported earlier [9] was that the red cell transketolase activity (TKA) level in pernicious anemia is distinctly increased. In the course of treatment, reticulocytosis increased and the TKA was gradually restored to normal. While the present series was being examined, some nonpernicious patients showed spurious TKA values. A closer examination revealed [8, 10] that these patients were affected with chronic nephropathy and uremia. In a later, somewhat more extensive series it was found that in uremia the red cell TKA may be remarkably high, yet within the range of normal variation. Extremely few values lower than the normal level may occur [10].

Recently, the problem has again attracted attention [3, 13]. LONERGAN *et al.* [5–7] and STERZEL *et al.* [14] reported that the red cell TKA was inhibited in uremia and that these patients had a plasma factor responsible for the inhibition of this red cell enzyme. This factor has also been considered to inhibit the transketolation of the central nervous system cells

[1] Grant from The Emil Aaltonen Foundation, Tampere, Finland.

[14]. The inhibition phenomenon has readily been associated with uremic neuropathy.

In the following uremic series, attention was devoted to the transketolase activity of red cells, and the presence of a possible inhibitory factor in the plasma of uremic patients.

Material and Methods

The series comprised a total of 336 subjects, of whom 286 were healthy controls (mean age 64 years, range 10–97 years, weight 60±15 kg, and Hb 13.7±1.9 g/100 ml). The uremic patients examined numbered 50 (mean age 48 years, range 19–81 years, 25 of either sex). The detailed characteristics concerning the uremic patients are given in table I.

The control subjects were healthy and clinical examination failed to reveal in them any disease which, according to currently held views, should affect the TKA of the red cells. In particular, their medical histories or clinical status showed no signs of renal insufficiency (serum creatinine, specific weights of morning urine, urinary sediment and hemoglobin).

All the uremic patients were also examined under clinical conditions at the University Department of Medicine by the kidney specialist (J.F.) of our team. All patients underwent a careful clinical examination, and also a controlled clinicochemical and roentgenological examination. To ascertain the renal status, special attention was given to the points mentioned in table I, which were closely observed. The diagnosis was based, besides the clinical examination, on renal biopsy and the histological picture so obtained. The etiology of the uremia was by this means traced back to chronic, acute or lupoid glomerulonephritis, chronic interstitial nephritis, chronic pyelonephritis, nephropathy of the diabetics, nephrocalcinosis, nephrosclerosis, bilateral polycystic kidney, amyloidosis of the kidneys, hypertension with (heart and) kidney disease, essential hypertension, agenesis of the kidneys, cancer of the prostate, and miscellaneous.

An experiment was made on an unselected group of 8 uremic patients (table IV), with serum creatinine and red cell TKA taken immediately before and immediately after dialysis. These patients had been on regular dialysis treatment for periods ranging from a few months to 3 years, for chronic renal insufficiency (chronic glomerulonephritis 4, chronic pyelonephritis 3, and polycystic degeneration of the kidneys 1 patient). The test dialysis lasted 10 h and the three-layer Kiil dialyser was used.

Another experiment was carried out to study the TKA inhibiting or activating qualities of the uremic serum. The red cell hemolysates of a healthy subject and of a uremic patient were mixed with saline, normal plasma and uremic plasma. The results of the experiment are presented in table V.

The samples for red cell TKA were drawn from venous blood, treated and determined as reported earlier [10].

The results of the study were computerized (Department of Applied Mathematics, University of Turku), and the output is shown in table I. In addition, a correla-

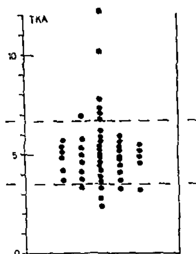


Fig 1 The individual values of red cell TKA in uremic patients (Minimum 2.4, maximum 12 2, and mean 5 1 units/ml) The broken lines show the area of normal variation in 286 healthy subjects

Table I The clinico-chemical characteristics of the uremic patients

	Creatinine, mg%	Urea, mg%	K, mmole/l	Na, mmole/l	Ca, mmole/l	Hb, g%	HCR, g%	Reticulocytes, %
Mean	6 1	50.2	4 2	137 3	4 6	10 1	32 3	1 7
SD	5 4	37 1	0 8	4 0	0 8	2.8	8 9	1 2
Sem	0 8	6 0	0 1	0 8	0 1	0 4	1 3	0 2
Min	1 6	13 4	3 1	118 0	3 1	4 8	14 0	0 5
Max	23 1	195 3	6 5	145 0	6 7	15 5	50 0	6 0

tion calculation was carried out, correlating the red cell TKA with the diagnosis, creatinine, urea, serum potassium, sodium and calcium, hemoglobin, hematocrit, reticulocyte count and bone marrow morphology

Results

The red cell TKA obtained for the 286 controls was 4 8 ± 0 7 units/ml (mean ± SD) (range 3 5–6 7) According to fig 1, the red cell TKA for

[14] The inhibition phenomenon has readily been associated with uremic neuropathy.

In the following uremic series, attention was devoted to the transketolase activity of red cells, and the presence of a possible inhibitory factor in the plasma of uremic patients.

Material and Methods

The series comprised a total of 336 subjects, of whom 286 were healthy controls (mean age 64 years, range 10–97 years, weight 60±15 kg, and Hb 13.7±1.9 g/100 ml). The uremic patients examined numbered 50 (mean age 48 years, range 19–81 years, 25 of either sex). The detailed characteristics concerning the uremic patients are given in table I.

The control subjects were healthy and clinical examination failed to reveal in them any disease which, according to currently held views, should affect the TKA of the red cells. In particular, their medical histories or clinical status showed no signs of renal insufficiency (serum creatinine, specific weights of morning urine, urinary sediment and hemoglobin).

All the uremic patients were also examined under clinical conditions at the University Department of Medicine by the kidney specialist (J. F.) of our team. All patients underwent a careful clinical examination, and also a controlled clinicochemical and roentgenological examination. To ascertain the renal status, special attention was given to the points mentioned in table I, which were closely observed. The diagnosis was based, besides the clinical examination, on renal biopsy and the histological picture so obtained. The etiology of the uremia was by this means traced back to chronic, acute or lupoid glomerulonephritis, chronic interstitial nephritis, chronic pyelonephritis, nephropathy of the diabetics, nephrocalcinosis, nephrosclerosis, bilateral polycystic kidney, amyloidosis of the kidneys, hypertension with (heart and) kidney disease, essential hypertension, agenesis of the kidneys, cancer of the prostate, and miscellaneous.

An experiment was made on an unselected group of 8 uremic patients (table IV), with serum creatinine and red cell TKA taken immediately before and immediately after dialysis. These patients had been on regular dialysis treatment for periods ranging from a few months to 3 years, for chronic renal insufficiency (chronic glomerulonephritis 4, chronic pyelonephritis 3, and polycystic degeneration of the kidneys 1 patient). The test dialysis lasted 10 h and the three-layer Kiil dialyser was used.

Another experiment was carried out to study the TKA inhibiting or activating qualities of the uremic serum. The red cell hemolysates of a healthy subject and of a uremic patient were mixed with saline, normal plasma and uremic plasma. The results of the experiment are presented in table V.

The samples for red cell TKA were drawn from venous blood, treated and determined as reported earlier [10].

The results of the study were computerized (Department of Applied Mathematics, University of Turku), and the output is shown in table I. In addition, a correla-

Table II Characteristics of the uremic patients whose TKA exceeds the normal variation

Patient No.	Diagnosis	Sex	Age, years	Creatinine mg%	Urea, mg%	Hb, g%	K, mmole/l	TKA, units/ml
1	chronic pyelonephritis	F	44	8 6	40 0	7 7	4 1	6 8
2	nephrosclerosis	M	25	12.8	82 9	6 2	4 4	6 9
3	glomerulonephritis	M	24	14 7	–	5 6	5 0	7 0
4	polycystic kidney, bilateral	F	52	9 4	52.2	11 9	4 3	7 4
5	polycystic kidney, bilateral	F	28	20 4	195 3	4 8	3 4	7 8
6	chronic pyelonephritis	F	42	13.2	68 6	5 8	6 5	10 2
7	chronic pyelonephritis	F	42	12 9	–	4 9	6 1	12 2

Table III Characteristics of the uremic patients whose TKA is below the normal variation

Patient No	Diagnosis	Sex	Age, years	Creatinine, mg%	Urea, mg%	Hb, g%	K, mmole/l	TKA, units/ml
1	chronic(?) pyelonephritis	F	65	1 6	–	12 9	4 9	2.8
2	(renal) hypertension	M	50	2 0	32.8	13 8	3 8	2 4
3	(renal) hypertension	M	47	5 0	28 6	8 2	4 2	3 3
4	chronic pyelonephritis	F	54	3 5	40 6	11.6	3 9	3 3
5	chronic glomerulonephritis	M	33	3 2	32.0	14 8	3 1	3 3
6	chronic pyelonephritis	F	35	1 8	27 4	13 0	3 3	3 4

nificant TKA is positively correlated with serum creatinine, urea and potassium levels, and negatively correlated with the hemoglobin and hematocrit of the uremic patients A 10-hour dialysis does not affect the red cell TKA, nor does uremic plasma contain factors inhibiting or activating

Table II. Serum creatinine and red cell TKA in 8 patients before and after 10-hour dialysis

Patient No.		Creatinine, mg%	TKA, units/ml
1	before dialysis	12.0	5.2
	after dialysis	7.0	4.9
2	before dialysis	11.0	12.5
	after dialysis	3.4	11.0
3	before dialysis	9.0	6.4
	after dialysis	4.6	5.8
4	before dialysis	13.0	5.5
	after dialysis	5.2	5.7
5	before dialysis	13.0	6.7
	after dialysis	7.1	6.9
6	before dialysis	14.5	10.3
	after dialysis	6.0	10.9
7	before dialysis	15.0	7.0
	after dialysis	7.8	6.8
8	before dialysis	13.8	4.3
	after dialysis	6.2	4.9

red cell TKA. These findings corroborated our earlier observations [8] according to which the red cell TKA in uremia was normal or occasionally higher than normal. JENNESKENS *et al.* [4] also found that the TKA was normal in uremia, although the patients were affected with neuritis. RIZZO and VARASI [13] found that the TKA in uremia was significantly higher than in the controls, but that in the former the so called TPP effect was low, indicating that uremia involved no thiamine deficiency. FERRONE *et al.* [1] reported that glutathione reductase in the red cells of uremic patients was increased, most markedly in those with anemia. The present observations supported this view. The activity of glucose 6-phosphate dehydrogenase, the key enzyme for pentose phosphate shunt, has by many authors been reported to have increased in the uremic patients [16].

Most of the above authors agree that, in uremia, the activity of the enzymes of pentose phosphate shunt is either normal or slightly increased. Recent literature, however, contains several mentions according to which the red cell TKA is reduced in uremia, specifically in certain parts of the

Table V Effect of saline, normal plasma and uremic plasma on the TKA of normal red cells and the red cells of the uremic patient TKA diluted 1 1

Hemolysate	0 9% saline	Normal plasma	Uremic plasma	TKA, units	Recovery, %
Normal erythrocytes					
2	2	–	–	2.84	100 0
2	1	1	–	2.84	100 0
2	–	2	–	2 79	98 3
2	1	–	1	2.79	98 3
2	–	–	2	2 90	102.0
Uremic erythrocytes					
2	2	–	–	7.25	100 0
2	1	1	–	7 25	100 0
2	–	2	–	7 13	98 3
2	1	–	1	7 42	102.3
2	–	–	2	6 96	96 1

central nervous system [14] These authors have combined this observation with uremic neuropathy and have even been able to show, in the serum of the uremic patient, a substance which reduces TKA The present studies, like many of those listed above, fail to corroborate this finding

According to the currently held view, reduced TKA values in the red cells can imply a deficiency of the cofactor, thiamine *For this reason, TKA determinations* are used as a measure of the thiamine deficiency at cellular level Reduced TKA values have been reported in nutritional disturbances in alcoholics [10] and in post operative conditions after intestinal resection [9] The red cell TKA is also low during the acute attacks in hepatic porphyria [10] It has also been discovered that, in patients taking diphenylhydantoin, some plasma factor, although not DPH alone, strongly inhibits the transketolase activity of red cells [11] In uremia, the *thiamine-pyrophosphate effect is low* [13] which means that the patients are not deficient in thiamine. This could actually be shown in the analytic studies of plasma [12] in which the plasma thiamine in uremia *proved to be higher than in healthy controls.* Hemodialysis produced no change in the situation [12] According to the present study there are no TKA inhibiting factors in the plasma of the uremic patients to prevent the customary use of thiamine pyrophosphate in enzyme reactions. Nor did those of the present patients who had a slightly reduced TKA, show any signs of uremic neuropathy Neither did the plasma contain any TKA activator

Although there is a tendency to associate the reduced TKA values with thiamine deficiency neuropathy *this need not necessarily* always be justified. The TKA in red cells may be normal although the patient already shows signs of neuritis, while on

the other hand the TKA may be reduced before any signs of neuritis are noticeable. This necessitates an evaluation of the neurological methods on the sensitivity of which the diagnosis of neuritis depends.

A preliminary report from our laboratory [9] indicated that the red cell TKA is definitely increased in pernicious anemia, an observation that has later been confirmed by WELLS *et al.* [15]. It is known that this anemia may possibly be accompanied by marked symptoms of neuropathy. In the present study, however, we were unable to observe any relationship between the degree of red cell TKA and the presence or absence of the neuropathy of pernicious anemia. In this same connection, it emerged, admittedly, that remarkably high TKA values may occur in uremia without pernicious anemia having been diagnosed by bone marrow examination or some other means. HAMPERS *et al.* [2] pointed out that a considerable proportion of uremic patients show low serum folic acid levels and that they have a tendency to 'varying degrees of megaloblastosis'.

Neuropathy, therefore, can occur in association with TKA values that are lower or higher than the normal. In the present series, the values were slightly above the average, but both low and quite high values were recorded. None of the examined subjects showed neuritis. In our opinion, the TKA level in uremia is in no way related to neuritis, but is more likely to involve a reactive change in the pentose phosphate pathway, intended e.g. to protect the red cell against oxidant agents. The activation of the enzymes of this shunt would thus be associated with a compensation process, also indicated by the distinct activation of glutathione reductase in uremia, especially in the anemic patients [1]. The present findings support this view.

References

1 FERRARI, S.; ZANELLA, A., and SIRCHIA, G.: Erythrocyte glutathione reductase activity in chronic renal disease. Scand. J. Haemat. 7: 409–412 (1970).
2 HAMPERS, C. L.; STREIFF, R.; NATHAN, D. G.; SNYDER, D., and MERRILL, J. P.: Megaloblastic hematopoiesis in uremia and in patients on long-term hemodialysis. New Engl. J. Med. 276: 551–554 (1967).
3 HENDERSON, L. W.: Erythrocyte transketolase inhibition, neuropathy and uremia. New Engl. J. Med. 284: 1435–1436 (1971).
4 JENNEKENS, F. G. I.; MEES, E. J. D., and VAN DER MOST VAN SPIJK, D.: Clinical aspects of uraemic polyneuropathy. Nephron 8: 414–426 (1971).
5 LONERGAN, E. T.; SEMAR, M., and LANGE, K.: Transketolase activity in uremia. Arch. intern. Med. 126: 851–854 (1970).

6 LONERGAN, E T, SEMAR, M, and LANGE, K A dialyzable toxic factor in uremia Trans amer Soc. artif intern. Organs *16*. 269–271 (1970)
7 LONERGAN, E T, SEMAR, M, STERZEL, R. B, TRESER, G, NEEDLE, M A., VOYLES, L, and LANGE, K. Erytrocyte transketolase activity in dialyzed patients A reversible metabolic lesion of uremia New Engl J Med *284* 1399–1403 (1971)
8 MARKKANEN, T Transketolase activity of red blood cells in conditions of haematological interest Acta haemat, Basel *39* 321–332 (1968)
9 MARKKANEN, T and KALLIOMAKI, J L. Transketolase activity of blood cells in various clinical conditions Amer J med. Sci. *252* 564–568 (1966)
10 MARKKANEN, T and PELTOLA, O Pentose phosphate pathway of erythrocytes in metabolic diseases Acta haemat., Basel *45* 285–289 (1971)
11 MARKKANEN, T, PELTOLA, O, HIMANEN, P, and RIEKKINEN, P Metabolite(s) of diphenylhydantoin in human plasma inhibit(s) the pentose phosphate pathway of leucocytes Pharmacology *6* 216–222 (1971)
12 NIWA, T ITO, T, MATSUI, E., and CITY, O Plasma thiamine levels with hemodialysis. J amer med Ass *218* 885–886 (1971)
13 RIZZO, S C and VARASI, C Valutazione dello stato nutritizionale tiaminico di soggetti uremici mediante determinazione dell attività transchetolasica eritrocitaria. Minerva. nefrol *16* 301–305 (1969)
14 STERZEL, R. B, SEMAR, M, LONERGAN, E T, TRESER, G, and LANGE, K. Relationship of nervous tissue transketolase to the neuropathy in chronic uremia J clin Invest *50* 2295–2304 (1971)
15 WELLS, D G, BAYLIS, E. M, HOLOWAY, L., and MARKS, V Erythrocyte-transketolase activity in megaloblastic anaemia Lancet *ii* 543–545 (1968)
16 VUOPIO, P Red cell enzymes in anemia. Scand. J clin. Lab Invest. *15*, suppl 72 (1963)

Authors address Assoc. Prof of Med T MARKKANEN, MD, O PELTOLA, MSc, J FORSSTRÖM, MD and P HIMANEN MD Vanha Littoistentie 50, *SF 20520 Turku 52* (Finland)

Acta Haemat. 48: 278–282 (1972)

Leucocyte Loss in Haemodialysis

L. Buscarini and F. Bassi

Medical Department, Hospital of Fiorenzuola d'Arda

Abstract. A sedimentation of leucocytes on the membranes of the Kiil kidney occurs during haemodialysis. The cells are preponderantly represented by granulocytes; they are lost at the end of dialysis. We calculated the number of lost leucocytes to be about 10×10^{8}.

Key Words
Haemodialysis
Leucocyte loss in haemodialysis
Uraemia

In 1968, Kaplow and Goffinet [3] described, early in haemodialysis, a transient neutropenia with fall of monocytes while the number of lymphocytes is the same. Gral *et al.* [2] confirmed the phenomenon with two other types of dialyzer. Brubaker and Nolph [1] pointed out that neutropenia does not depend on definitive loss in the circulating pool of granulocytes and observed a re-appearance of the labeled cells. Moreover the period of neutropenia is followed by an influx of neutrophils from the bone marrow. The neutropenic phase appears 10–20 min after the beginning of haemodialysis. The recovery is accomplished in the time which immediately follows. In 1.5–3.5 h the neutrophil count rises to a higher value than the starting values (sometimes it is nearly double).

Toren *et al.* [4] postulated that a humoural factor formed in the dialyzer causes perhaps the granulocytic sequestration in the lungs. The phenomenon was also ascribed to a pulmonary sequestration of neutrophils damaged by passing in the coil; a secondary agglutination of all other granulocytes could occur [1].

During researches on this problem we have observed on the membranes of Kiil kidney a remarkable amount of leucocyte sediments during haemodialysis; they are lost at the end of dialysis.



Fig 1a Fragment of cuprophan membrane drawn at the end of an 11 hour haemodialysis, stained with May-Grunwald-Giemsa. *b, c* The dark bands are constituted by sedimented leucocytes.

Methods

At the end of an 11 hour haemodialysis we have drawn fragments of membrane in different sectors of dialyzer These fragments, stretched on slides, were stained

Thus, it is likely that the loss of leucocytes in every haemodialysis is much more important than we suggested (10×10^9 The phenomenon could have practical implications

References

1 BRUBAKER, H. L. and NOLPH, K. D. Mechanisms of recovery from neutropenia induced by hemodialysis. Blood 37: 623 (1971)
2 GRAL, T., SCHROTH, P., PALMA, J. R. DE, and GORDON, A. Leucocyte dynamics with three types of hemodialyzers. Trans. amer. Soc. artif. intern. Organs 15: 45 (1969)
3 KAPLOW, L. S. and GOFFINET, J. A. Profound neutropenia during the early phase of haemodialysis. J. amer. med. Ass. 203: 133 (1968)
4 TOREN, M., GOFFINET, J. A., and KAPLOW, L. S. Pulmonary bed sequestration of neutrophils during haemodialysis. Blood 36: 337 (1970)

Authors' address: Dr. LUIGI BUSCARINI and Dr. FRANCESCO BASSI, Medical Department, Hospital of Fiorenzuola d'Arda, 29017 Fiorenzuola d'Arda (Italy)

Acta Haemat 48. 283-287 (1972)

Cytogenetic Observations on Different Cell Lines in Hodgkin's Disease

C. D Olinici

Institute of Oncology (Director Prof Dr I Kiricuta), Cluj

Abstract A case of Hodgkin's disease is presented, where cytogenetic examination has detected the presence of 3 cell lines. It is suggested that hypotriploid metaphases could originate in Hodgkin's cells and diploid metaphases in lymphocytes Pseudodiploid cells (which present a cytogenetic constitution suggesting a clonal origin) could derive from a population of neoplastic lymphocytes

Key Words
Cell lines
Hodgkin's disease
Karyotype

In a recent paper, Spriggs [12] has reviewed the main cytogenetic and microspectrophotometric data which suggest the malignant nature and the clonal origin of Hodgkin's cells However, it is not clear if the Hodgkin's cells are the only malignant cells, the other cell types which constitute the histologic picture of Hodgkin's disease being reactive elements or residues of the original lymphatic tissue Based on histopathologic (the presence of lymphocytes in the metastases of Hodgkin's disease, the proliferation of immature lymphoid cells) and clinical observations (the association between Hodgkin's disease and lymphocytic leukemia), Willis [14] and Evans [3] believe that some lymphoid cells could have a neoplastic character The case we present here supports this point of view

Case Report

The patient, a woman aged 34, was first admitted in May 3, 1968 Histopathologic examination of a cervical lymph node revealed a Hodgkin's disease The patient was irradiated with 16 000 rad between May 3, 1968 and June 28, 1968,

1 —2— —3— —4-5—

6 — XX — 12

—13 - 15— —16 - 18—

— 19-20 — — 21 - 22 — M1 M2

Fig 1 Karyotype of a metaphase with 65 chromosomes. There are 2 marker chromosomes a long submetacentric chromosome (M_1) and a long acrocentric chromosome (M_2)

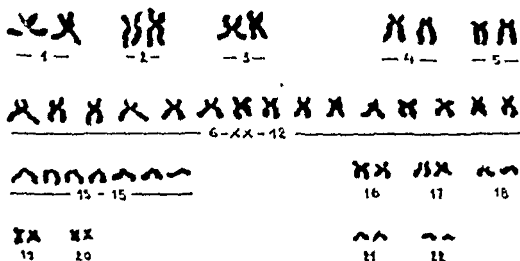


Fig 2 Karyotype of a pseudodiploid cell 46,X (-,D)+

Table I Chromosome counts in 39 cells

Chromosomes	45	46	47	63	65	66	67	68	69	138	140
Number of cells	3	15	2	1	1	4	3	4	1	1	1

5,400 rad between November 13 and December 21, 1968, and 11,200 rad between January 11 and March 26, 1971

She was hospitalized again in July 8, 1971, this time with a generalized disease, the mediastinal and mesenteric lymph nodes, the spleen and the liver were involved, and a moderate amount of serous fluid was found in the peritoneal cavity At the cytologic examination of the ascitic effusion, numerous lymphocytes, Hodgkin's cells, and a few Sternberg Reed cells were identified

Chromosomes in the cells of the ascitic effusion were prepared by the direct method of SLOT [10] 36 cells were exactly counted, chromosome numbers are shown in table I Two peaks were observed, one at 46 chromosomes, the other at 66–68 chromosomes Hypotriploid cells contain 2 marker chromosomes a subme [illegible] sized [illegible] M_2- [illegible]

13 cells with 46 chromosomes were karyotyped Six cells had a normal karyotype, one cell showed an additional acentric fragment, 6 cells were pseudodiploid one of them had a 46,XX,3–, F+ karyotype, the other 5 cells were 46,XX,C–,D+ (fig 2) No consistent chromosome losses were observed in the cells with 45 chromosomes

Discussion

The occurrence of 2 cell lines is not an unusual finding in Hodgkin's disease [1, 6–9, 11] One of these cell lines is characterized by a chromosome number in triploid or tetraploid range Evidence has been presented that high DNA contents are characteristic for Hodgkin's cells [7] Cytogenetic features of these cells suggest their clonal origin [12], evident in our case, too In some cases, marker chromosomes were observed [2, 4, 6, 9, 13, present case], their morphological aspects were quite variable and failed to indicate any specificity

The second cell line is represented by cells in diploid range The majority of the metaphases have a normal karyotype and are probably derived from lymphoid or plasma cells [2, 12] However, some of the metaphases with 46 chromosomes may have a pseudodiploid karyotype

[6–9, 11]. Hyperdiploid cells can be observed too [11], and PECKHAM and COOPER [7] have shown that a small proportion of cells previously identified as transformed lymphocytes, presented aneuploid DNA values.

In our case, the cytogenetic examination has revealed the presence of 3 cell lines: hypotriploid, diploid and pseudodiploid cells. The first class in probably represented by Hodgkin's cells, and the second by normal lymphoid cells. We suggest that pseudodiploid metaphases (which show a cytogenetic constitution highly suggesting a clonal origin) may have been derived from a population of neoplastic lymphocytes. If the existence of neoplastic lymphocytes in Hodgkin's disease would be confirmed, it would be extremly improbable that they occur only in those cases which present cytogenetic abnormalities in the peri-diploid cells. It is possible that some apparently diploid cells were neoplastic too, as it has been suggested by SPIERS and BAIKIE [11]. In this case, pseudodiploid or hyperdiploid cells could represent the result of a clonal evolution, similar to the commonly observed process in chronic granulocytic leukemia. The hypothesis of KAPLAN [5] on the role of immunologic disturbance in Hodgkin's disease is in accord with our data. The author supposes that 'truly neoplastic lymphoid cells might evolve as the end-stage consequence of an initially non-neoplastic viral infection capable of inducing antigenic alteration (and perhaps also chromosomal instability) in a subpopulation of lymphocytes'.

Neither in our case, nor in the cases published by others, has a correlation been observed between the different cell lines in Hodgkin's disease. This finding does not agree with the hypothesis of a common origin, from a primitive reticular cell, of both Hodgkin's cells and presumably malignant lymphocytes [3, 14]. It is possible that the neoplastic stimulus may act on more differentiated cells, the two series being independently affected.

The available data do not permit firm conclusions to be drawn. Other studies are necessary in order to confirm or to invalidate the existence of neoplastic lymphoid cells, a problem with major implications for the staging and for the therapy of Hodgkin's disease.

References

1 BAKER, M. C. and ATKIN, N. B.: Chromosomes in short term cultures of lymphoid tissue from patients with reticulosis. Brit. med. J. i: 770–771 (1965).

2 COUTINHO, V, BOTTURA, C., and FALCAO, R. P. Cytogenetic studies in malig nant lymphomas a study of 28 cases Brit. J Cancer *25* 789–801 (1972)
3 EVANS, R. W Histological appearance of tumours, 2nd ed., p 252 (Living stone, Edinburgh 1968)
4 GALAN H M, LIDA, E. J., and KLEISNER, E H Chromosomes of Sternberg-Reed cells. Lancet *i* 335 (1963)
5 KAPLAN, H S Role of immunologic disturbance in human oncogenesis some facts and fancies. Brit J Cancer *25* 620–634 (1972)
6 MILES C P, GELLER, W., and O'NEILL, F Chromosomes in Hodgkin's dis-ease and other malignant lymphomas Cancer *19* 1103–1116 (1966)
7 PECKHAM, M J and COOPER, E H Proliferation characteristics of the various classes of cells in Hodgkin's disease Cancer *24* 135–146 (1969)
8 RICCI, N, PUNTURIERI, E, BOSI L., and CASTOLDI, G L. Chromosomes of Sternberg Reed cells Lancet *ii* 564 (1963)
9 SEIF, G S. F and SPRIGGS, A. I Chromosome changes in Hodgkin's disease J nat. Cancer Inst. *39* 557–570 (1967)
10 SLOT, E A karyologic study of the cancer of the ovary and the cancer cells in the ascitic effusions Neoplasma *14* 2 10 (1967)
11 SPIERS A. S. D and BAIKIE, A G Cytogenetic studies in the malignant lym phomas and related neoplasms Results in twenty-seven cases. Cancer *22* 193–217 (1968)
12 SPRIGGS, A. I Clonal proliferation in Hodgkin's disease Lancet *i*. 857 (1971)
13 SPRIGGS A. I. and BODDINGTON M M Chromosomes of Sternberg Reed cells. Lancet *ii* 153 (1962)
14 WILLIS, R. A. Pathology of tumours 4th ed., pp 783–789 (Butterworth, Lon don 1967)

Author's address Dr C. D OLINICI, Institute of Oncology, Republicii 34–36, *Cluj* (Romania)

Acta Haemat. 48: 288–291 (1972)

Ammoniacal Silver Staining of Reed-Sternberg Cells in Hodgkin's Disease[1]

L. Kass and B. Schnitzler

Departments of Internal Medicine (Simpson Memorial Institute) and Pathology, The University of Michigan, Ann Arbor, Mich.

Abstract. When stained with the ammoniacal silver stain, Reed-Sternberg cells in lymph nodes obtained from 6 patients with Hodgkin's disease present a unique appearance. They appear as large multinucleated cells with orange brown nucleoli and bright yellow cytoplasm containing brown-staining granules. Malignant histiocytes and bizarre appearing reticulum cells in Hodgkin's disease and other lymphomas did not exhibit these staining properties.

Key Words
Hodgkin's disease
Reed-Sternberg cells
Silver staining

The present studies describe cytochemical properties of Reed-Sternberg cells using the ammoniacal silver stain (AS).

Materials and Methods

Lymph node biopsies obtained from patients with a variety of lymphomas, including 4 patients with lymphocytic lymphoma, 3 patients with reticulum cell sarcoma, and 6 patients with Hodgkin's disease, were fixed for 48 h in acetate buffered formalin. The tissue was processed in the usual manner, imbedded in paraffin, sectioned 4-5 μm thick, and deparaffinized. The sections were stained with AS stain [1] and mounted with Permount on glass slides for microscopic examination. Serial sections were stained with hematoxylin and eosin for comparison with the AS-stained slides.

[1] Supported in part by American Cancer Society Institutional Research Grant IN-40-K, The University of Michigan Cancer Research Institute.

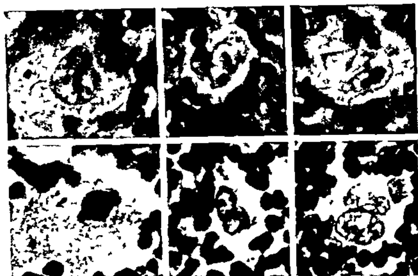


Fig 1 Top row Hematoxylin eosin stained Reed Sternberg cells. Bottom row AS-stained Reed-Sternberg cells in serial section corresponding to the cell above it in the top row

Results

Typical Reed Sternberg cells stained with hematoxylin and eosin were identified according to the criteria of RAPPAPORT [2] They are illustrated in the top row of figure 1 A serial section was used for the AS stain as seen in the bottom row of figure 1 Cells corresponding to the Reed Sternberg cells on the HE section had a characteristic appearance on the AS-stained slide They had yellow staining vesicular nuclei with brown filamentous strands traversing them The cytoplasm stained intensely yellow and sometimes contained tiny brown staining granules Nucleoli stained bright orange brown Lymphocyte nuclei appeared dark brown in color with block like areas in the nuclei. No cytoplasmic staining was seen

Reticulum cells in reticulum cell sarcoma and poorly differentiated lymphocytes in lymphosarcoma did not exhibit the staining properties observed in Reed Sternberg cells Bizarre reticulum cells in certain patients with Hodgkin s disease (reticular type) had gray brown staining cyto-

plasm when stained with AS, but their nuclear staining properties resembled those of Reed-Sternberg cells.

Discussion

Identification of Reed-Sternberg cells in an appropriate cellular environment is considered to be essential for a definite diagnosis of Hodgkin's disease. These cells are generally readily identified when observed. However, the problem often lies in detecting the occasional Reed-Sternberg cell present in the biopsies of a patient with suspected Hodgkin's disease

Several investigators have proposed cytochemical techniques in an attempt to facilitate the identification of Reed-Sternberg cells. MENDELER and GREENWOOD [3] demonstrated that amido black 10B selectively stains nucleoli of both Reed-Sternberg cells and other bizarre-appearing mononuclear cells. Cytoplasmic pyrininophilia [1], PAS and aldehyde fuchsin positive cytoplasmic granules [5], and increased ribonucleoprotein and alkaline phosphatase [6] have been described as characteristic of Reed-Sternberg cells when compared to reticulum cells.

Our studies using the AS reaction demonstrate both cytoplasmic and nucleolar staining properties which appear to show specificity for the Reed-Sternberg cell

Aside from facilitating identification of the Reed-Sternberg cell, the AS reaction raises questions as to the pathogenesis of the staining reaction peculiar to this cell The AS stain is said to be specific for nucleohistones and to differentiate between lysine-rich and arginine-rich histone on the basis of color reaction [1] Lysine-rich histone stains yellow whereas arginine-rich histone stains brown to black Prior exposure of the specimen to acetate-buffered formalin is essential for success of the staining reaction

As far as hematological cells are concerned, we have observed intense yellow cytoplasmic staining as seen in the Reed-Sternberg cell only in megakaryocytes, proerythroblasts, and plasma cells It is conceivable that the yellow cytoplasm of these cells and of the Reed-Sternberg cell may result from an accumulation of histone in the cytoplasm of these cells

At times, Reed-Sternberg cells may resemble megakaryocytes, especially on sections stained with hematoxylin and eosin Using the AS stain, megakaryocyte cytoplasm stains grayish-brown in contrast to the intense yellow to yellow brown color of the Reed Sternberg cell cytoplasm The

Reed Sternberg cell nucleolus is deep orange using the AS stain, whereas the megakaryocyte usually does not have a nucleolus which can be visualized with certainty with the AS stain

References

1 Black, M M and Ansley, H R Antigen induced changes in lymphoid cell histones. I Thymus J Cell Biol 26. 201 (1965)
2 Rappaport, H A Tumors of the hematopoietic system p 156 (Armed Forces Institute of Pathology, Washington 1966)
3 Mundkur, B and Greenwood H Amido black 10B as a nucleolar stain for lymph nodes in Hodgkin s disease Acta cytol *12* 218 (1968)
4 Lukes, R. Criteria for involvement of lymph node, bone marrow, spleen, and liver in Hodgkin s disease Cancer Res *31* 1755 (1971)
5 Andritsakis, G D and Sommers, S S Criteria of thymic cancer and clinical correlations of thymic tumors. J thorac Surg *37* 273 (1959)
6 Ackerman, G A., Knouff, R. A., and Hoster, H A Cytochemistry and morphology of neoplastic and non neoplastic human lymph node cells with special reference to Hodgkin s disease J nat Cancer Inst. *12* 465 (1951)

Authors address Dr Lawrence Kass and Dr Bertram Schnitzer Department of Internal Medicine, Simpson Memorial Institute, University of Michigan, *Ann Arbor, MI 48104* (USA)

Acta Haemat. 48: 292-299 (1972)

Essais de standardisation d'un test de pression de filtration des éléments figurés du sang et application à l'agrégation plaquettaire[1]

J. F. Stoltz, A. Larcan, F. Streiff et A. Nicolas

Groupe de Recherches Hémorhéologiques, CRTS, Nancy

Abstract. The authors describe the standardization of a new technique for studying platelet aggregation, based on the filtration pressure exerted by aggregates on a given filter. First results concern the study of the filtration pressure of whole blood, platelet enriched plasma and the effect of ADP on concentrations ranging from 0.1 to 100 µM. The filtration pressure of PRP increases with the dose of ADP used. Biological and clinical applications of the method are considered both for aggregates of cells and for substances preventing aggregation.

Key Words
Screen filtration pressure
Platelet aggregation
Surface charge

Parmi les nombreuses techniques qui ont été décrites pour étudier les phénomènes d'agrégation plaquettaire, les plus connues utilisent la variation de transmission optique au travers d'une suspension colloïdale. La mobilité électrophorétique constitue un moyen indirect d'étude de cette agrégation; c'est ainsi qu'il a pu être vérifié qu'une mobilité diminuée coïncide le plus souvent avec une agrégation accrue. Enfin, la technique de filtration sous pression, mise au point par Swank *et al.* [18] constitue la dernière en date de ces techniques.

La méthode d'étude de l'agrégation plaquettaire qui reste la plus employée est celle décrite par Born et Cross [1] ou celles qui en dérivent [2, 12, 19]. Elle étudie les variations de l'intensité lumineuse d'un faisceau traversant une solution plus ou moins riche en thrombocytes ou en agrégats plaquettaires.

Une autre technique est l'électrophorèse en phase liquide [9]. Elle est basée sur le fait que les éléments figurés du sang en milieu physiologique

[1] Ce travail a été réalisé avec l'aide de la DRME (section Biologie), contrat No 72 34 234 00 480 75 01.

ont une charge superficielle negative et que l'agregation ne peut se pro duire que par une diminution du potentiel interfacial [4, 10, 13, 14] La methode de filtration sous pression, utilisee par SWANK *et al* [18] des 1964 fut parfois appliquee a l etude de l agregation plaquettaire [3, 15], du sang total [10, 16, 17] et du sang conserve [8] L'etude de la pression de filtration dans certains cas cliniques a fait l'objet de quelques etudes [5, 17] alors que HORNSTRA [6, 7] applique une technique semblable, *in vivo*, chez l'animal.

Nous rapportons ici nos premiers resultats concernant les essais de standardisation de cette technique et son application a l'etude de l'agregation plaquettaire

Methode

Principe Cette technique consiste à mesurer la pression induite par une suspension cellulaire traversant un filtre parfaitement calibré, avec un débit constant. Cette pression est transformée en impulsions electriques par une jauge de con trainte reliee a un enregistreur

Description de l'appareil L appareil de mesure de pression de filtration utilisé (fig 1) se compose (1) d un reglage de zero (2) d un reglage de gain (3) d'un bouton «start» pour la mise en marche du piston (4) d un bouton «reverse» per mettant de changer le sens de marche du piston, (5) d un bloc de mesure

Le bloc de mesure est constitue par (a) un support portant la seringue conte nant l echantillon à analyser (b) un capillaire avec un support de fixation du fil tre (c) un robinet à 3 voies permettant, selon sa position, l etalonnage de la jauge de contrainte, le rinçage des capillaires ou la transmission de pression s exerçant au niveau du filtre (d) une jauge de contrainte pourvue d un robinet à 2 voies permettant soit le remplissage de la jauge par de l eau physiologique soit le rac cord au manomètre mesurant la pression d etalonnage

L appareil est relié à un enregistreur permettant de travailler sur un grand nombre d echelles de mesures (echelles de voltage à partir de 20 μV et échelles d intensité à partir de 20 μA) et avec une gamme de vitesse de deroulement du papier variant de 1 à 1000 mm/mm

Matériel Les filtres utilisés (fig 2) sont métalliques, a pores cubiques de 20 μ d arete (Buckbee Mears Company, St Paul, Minn)

Le sang total provient de donneurs de sang du CRTS et a eté preleve sur citrate de sodium (3 8‰) en flacons silicones

Les PRP, obtenus par centrifugation du sang total a 25 °C pendant 10 min, ont été conservés en flacons silicones a temperature ambiante.

L ADP utilisée (Sigma) est preparee sous forme d une solution mere a 1 mg/ml (conservee à –80 °C) et qui est diluee au moment de l emploi

Etalonnage de l'appareil Au debut de chaque série de mesures, on pratique un etalonnage de l appareil Il consiste à exercer une pression à l aide d un manome

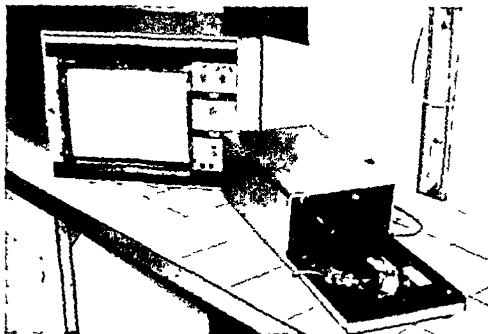


Fig. 1 Ensemble de filtration

tre à tension et à mesurer celle-ci par l'intermédiaire d'un manomètre à mercure. Le calibrage de l'échelle de l'enregistreur étant effectué, on réalise un test de linéarité entre la pression et l'enregistrement. Les premières mesures sont effectuées avec de l'eau physiologique, ce qui permet de vérifier la justesse de l'étalonnage et la propreté du filtre. Il faut éviter la présence de bulles d'air à l'intérieur des capillaires, celles-ci provoquant un accroissement de pression au niveau du filtre.

Standardisation des mesures. Nous avons utilisé deux anticoagulants : l'ACD et le citrate de sodium. Il ne semble pas y avoir de variations de la pression de filtration en fonction de ce facteur. Cependant, dans un souci de standardisation, nous avons choisi le citrate de sodium comme anticoagulant pour toutes nos études.

Les suspensions de mesure ont toujours été conservées à la température ambiante, car nous avons constaté qu'un sang total placé quelques heures à 4 °C donnait une pression de filtration nettement plus élevée que celle du sang conservé à température ambiante. Dans les mêmes conditions, les valeurs pour un PRP sont également très élevées. Il semble donc qu'un abaissement de température favorise l'agrégation des éléments figurés.

De plus, la pression de filtration d'un sang total est variable en fonction de son temps de conservation. C'est pourquoi les mesures ont toujours été réalisées le plus précocement après le prélèvement (moins de 4 h après le prélèvement).

En ce qui concerne le matériel utilisé, les premières mesures effectuées avec une verrerie ordinaire, se sont révélées inconstantes du fait d'une adhérence et

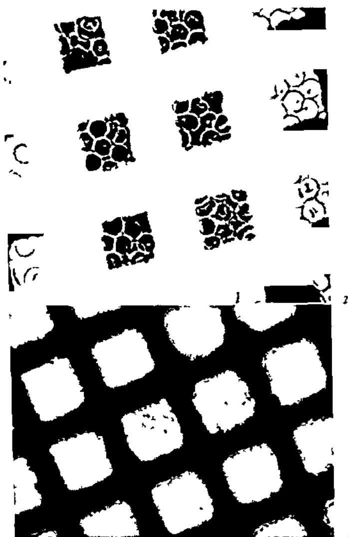


Fig 2 Agregats physiologiques d hématies
Fig 3 Agregats de plaquettes à l ADP

d une agrégation plus ou moins rapide et importante des elements figures. Aussi, opérons nous actuellement avec du materiel siliconé ou plastique.

Nous avons alors choisi des conditions de mesures toujours ident sant comme anticoagulant le citrate de sodium, en opérant



Fig. 1. Ensemble de filtration

tre à tension et à mesurer celle-ci par l'intermédiaire d'un manomètre à mercure. Le calibrage de l'échelle de l'enregistreur étant effectué, on réalise un test de linéarité entre la pression et l'enregistrement. Les premières mesures sont effectuées avec de l'eau physiologique, ce qui permet de vérifier la justesse de l'étalonnage et la propreté du filtre. Il faut éviter la présence de bulles d'air à l'intérieur des capillaires, celles-ci provoquant un accroissement de pression au niveau du filtre.

Standardisation des mesures. Nous avons utilisé deux anticoagulants : l'ACD et le citrate de sodium. Il ne semble pas y avoir de variations de la pression de filtration en fonction de ce facteur. Cependant, dans un souci de standardisation, nous avons choisi le citrate de sodium comme anticoagulant pour toutes nos études.

Les suspensions de mesure ont toujours été conservées à la température ambiante, car nous avons constaté qu'un sang total placé quelques heures à 4 °C donnait une pression de filtration nettement plus élevée que celle du sang conservé à température ambiante. Dans les mêmes conditions, les valeurs pour un PRP sont également très élevées. Il semble donc qu'un abaissement de température favorise l'agrégation des éléments figurés.

De plus, la pression de filtration d'un sang total est variable en fonction de son temps de conservation. C'est pourquoi les mesures ont toujours été réalisées le plus précocement après le prélèvement (moins de 4 h après le prélèvement).

En ce qui concerne le matériel utilisé, les premières mesures effectuées avec une verrerie ordinaire, se sont révélées inconstantes du fait d'une adhésivité et

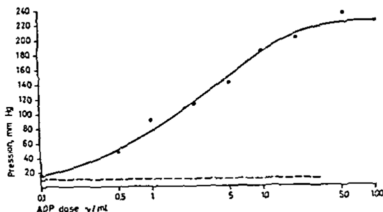


Fig 4 Action de l ADP sur la pression des plaquettes (filtre de 20 μm) Chaque point correspond à la moyenne de 8 experiences ---- Valeurs normales de la pression de filtration du PRP (7,8±4 2 mmHg).

Les PPP, obtenus par centrifugation a 3000 g de PRP, ont une pression de filtration inferieure à celle du PRP (4,2 mm Hg) Cette pression traduit la perte de charge du milieu plasmatique seul a travers les filtres

Nos resultats sont en bon accord avec ceux de SWANK [15] qui trouve que les valeurs normales de la pression de filtration du sang total immediatement apres le prelevement varient de 20 à 45 mm Hg.

En ce qui concerne les variations en fonction de l'hématocrite, les experiences de SWANK et DAVIS [16] sur des sangs ajustes à un hematocrite donne par leur propre plasma citrate ou heparıné, puis filtres sur Dacron avant mesure de la pression de filtration montrent une variation linéaire pour des hematocrites variant de 30 à 50 Il est cependant important de noter que le sang est filtré en ce qui concerne les experiences de SWANK alors que nous avons utilise quant a nous le sang total

DHALL et MATHESON [3] quant a eux, ont egalement etabli quelques resultats quantitatifs Opérant dans des conditions tres particulieres (en presence d ADP), ils trouvent une valeur normale de 46 mm Hg Etudiant de meme l influence de l hematocrite à partir de globules rouges ajustes par du PPP et en presence de PRP et d ADP, ils constatent une diminution de la pression de filtration avec l augmentation de l hematocrite Ils ne réussissent cependant pas à etablir une relation entre la pression de filtration et le nombre de plaquettes de sangs totaux

Pression de filtration et agregation plaquettaire Nous avons alors cherche à appliquer cette technique a l'etude de l'agregation des plaquet-

Tableau I Valeurs normales de la pression de filtration à 25°C pour le sang total, le PRP et le PPP

Hématocrite, %	Nombre de mesures	Pression de filtration, mm Hg
30	2	22 ± 10
31	2	23 ± 14
32	6	21 ± 11
33	7	27 ± 13
34	13	33 ± 14
35	25	30 ± 13
36	18	32 ± 13
37	30	29 ± 14
38	32	32 ± 13
39	16	30 ± 12
40	19	32 ± 12
41	12	35 ± 18
42	16	36 ± 18
43	11	40 ± 19
44	5	41 ± 20
PRP	55	7,8 ± 4,3
PPP	16	4,2 ± 0,8

ambiante, en pratiquant des mesures immédiates, en employant une verrerie siliconée ou plastique, en effectuant un changement de filtre à chaque mesure réalisée sur une prise d'essai constante (3 ml).

Résultats

Valeurs normales de la pression de filtration pour un sang total ou un PRP. Dans les conditions ainsi définies, les valeurs de la pression de filtration varient de façon importante en fonction de l'hématocrite (tabl. I). Cependant, bien que les expériences aient porté sur 214 échantillons différents, nous ne pouvons actuellement proposer une corrélation entre l'hématocrite et la pression de filtration dont la moyenne varie de 20 à 42 mm Hg.

En ce qui concerne le PRP (tabl. I), on constate une grande constance de la pression de filtration (7,8 mm Hg en moyenne sur 55 expériences) sans qu'aucune corrélation ne puisse être dégagée entre le nombre des plaquettes et la pression de filtration.

tes. En effet, on pouvait supposer que les produits agrégants diminuant la charge superficielle et entraînant la formation d'agrégats allaient provoquer une élévation de la pression de filtration par colmatage des filtres (fig. 2, 3). Nous avons utilisé l'ADP à des doses allant de 0,1 γ à 100 γ ml.

A 4 ml de PRP, on ajoute alors la quantité voulue d'ADP (temps 0). On agite et au temps t = 1 mn, on aspire le PRP dans la seringue de mesure et le volume est ajusté à 3 ml. La pression de filtration est alors mesurée. On constate alors que l'agrégation créée en présence d'ADP est fonction de la dose (fig. 4) et qu'il y a une augmentation de la pression de filtration avec la dose, avec une stabilisation pour des concentrations supérieures à 20 γ ml.

Il est bon, cependant, de remarquer que la pression de filtration pour les fortes concentrations d'ADP (10 à 100 γ ml) semble être influencée par la taille des agrégats (colmatage et saturation du filtre plus ou moins rapide) et par la sédimentation dans la seringue au moment de la mesure, entraînant ainsi une plus grande dispersion des résultats.

Conclusion

On peut donc penser que la technique de filtration est, par sa sensibilité et sa simplicité, une méthode très efficace d'étude de l'agrégation des éléments figurés et des plaquettes en particulier. Le domaine d'application pourrait alors s'étendre à la pathologie, à l'étude de la conservation et de la transfusion du sang ainsi qu'à l'exploration des propriétés pharmacologiques des drogues médicamenteuses tant sur le plan de l'agrégation que sur celui de la désagrégation.

Résumé

Les auteurs décrivent la standardisation d'une nouvelle technique d'étude de l'agrégation plaquettaire, basée sur la pression de filtration exercée par les agrégats au niveau d'un filtre déterminé. Les premiers résultats portent sur l'étude de la pression de filtration de sangs totaux, de plasmas riches en plaquettes et sur l'action de l'ADP à des concentrations variant de 0,1 à 100 γ ml. C'est ainsi que la pression de filtration d'un PRP en présence d'ADP croît en fonction des doses d'ADP utilisées. Les applications biologiques et cliniques sont envisagées tant dans le domaine de l'agrégation des éléments figurés que dans celui des substances désagrégeantes.

Materials and Methods

Twenty male rats of the Sprague-Dawley strain were used Their mean weight was 288 g (range 220–360 g) The animals were divided into 4 equal groups

Group 1 5 mg of testosterone propionate were injected subcutaneously into each animal daily for 8 days. On the 8th day X ray treatment was given as below, following which the hormone was continued for another 8 days

Group 2 Following initial X ray treatment testosterone propionate was injected in the same dose routine as in group 1 for 8 days.

Group 3 One control group received X ray treatment without androgen

Group 4 A second control group received neither X ray nor hormonal treatment

White blood counts, hematocrits, and differential counts were performed on rat tail blood (by clipping) in all 4 groups before initiation of treatment, and then on days 3, 6, and 8 after X ray therapy On the 9th post irradiation day ^{59}Fe and $^{99}Tc^{m}$-sulfur colloid were administered intravenously as will be further described, final sacrifice by exsanguination being followed by separation, cleaning, and placing of both tibiae and femurs into isotope counting vials.

X ray treatment A 300-kV X ray machine with HVL of 2.0 Cu and SSD of 50 cm was used The output was 150 R/min. The entire left femur and tibia of each rat were shielded by 1.0 cm of lead Before irradiation each animal was anesthetized with nembutal The X ray exposure given was 800 R in one sitting

Isotopic studies Bone marrow reticuloendothelial and erythroid function was studied by the tracers technetium 99-sulfur colloid and iron 59 (ferrous) citrate (Abbott Radiopharmaceuticals) respectively Four hours prior to sacrifice 10 μCi ^{59}Fe as ferrous citrate of high specific activity (approximately 25 μCi/μg) were injected into a lateral ear vein One half hour prior sacrifice 0.5 mCi of $^{99}Tc^{m}$-sulfur colloid were similarly injected into each animal. $^{99}Tc^{m}$ was eluted from a commercial generator (Amersham/Searle Radiopharmaceuticals) and the sulfur colloid was prepared immediately thereafter by a standard thiosulfate technique using albumin as stabilizer At sacrifice, 10 ml whole blood were removed from the inferior vena cava to check residual circulating activity, and isotope content of alternate right and left tibia and femur was assayed using 1- and 10-min counts for $^{99}Tc^{m}$ and ^{59}Fe, respectively and full peak window with a single channel analyzer and a 2 in $\times$ 2 in NaI (Tl) crystal at a distance of 12 in from the specimens to minimize geometry effects. The radiation effect was expressed as a ratio of right-to-left counts after background subtraction

Results

The absolute numbers of peripheral white blood cells, polymorphonuclear leukocytes, and lymphocytes for all 4 experimental groups are summarized in table I The results demonstrate a slight increase from $17{,}380 \pm 800/mm^3$ to $19{,}700 \pm 1{,}130/mm^3$ in the total white blood cells

Acta Haemat. 48: 300–306 (1972)

The Effect of Androgens on Erythroid and Granulocytic Marrow Recovery in the Irradiated Rat

Y. Horn[1] and D. C. Price

Departments of Medicine and Radiology (Nuclear Medicine Section), University of California, San Francisco, Calif.

Abstract. A study was performed on the effect of testosterone on post-irradiation recovery of bone marrow and circulating granulocytes in the rat. Testosterone accelerated recovery of peripheral polymorphonuclear leukocytes, more so if therapy was initiated prior to irradiation. Increased marrow ^{59}Fe compared to controls confirmed the simultaneous erythroid stimulating effect of testosterone. ^{99m}Tc-sulfur colloid uptake in the marrow was also noted to be increased in all irradiated groups without a differential testosterone effect, perhaps reflecting a post-irradiation recovery response of increased vascularity. The possible mechanisms are discussed.

Key Words: Bone marrow · Erythropoiesis · Leukopoiesis · Rat bone marrow · Testosterone

Androgens are known to accelerate the post-irradiation recovery of erythroid bone marrow [8]. Acceleration of the recovery of the circulating leukocytes by androgens has been described in cancer patients [14] and in irradiated rats [9, 12]. This leukopoietic effect is sex- and dose-dependent and also correlates with the anabolic or androgenic activity of the given steroid hormone [10, 11].

The erythropoietic stimulatory effect of androgens is known to be mediated through erythropoietin [4]. Less is known about the leukopoietic mediators, although there are various postulations concerning the existence of a humoral leukopoietic factor [3, 5–7, 17]. It is also not known if the combined post-irradiation effect of androgens is mediated via a common or via separate erythroid and leukocytic mechanisms.

The purpose of the present paper is to try to clarify the effect of testosterone propionate on both the white and the red cells in the bone marrow of irradiated rats as to its erythropoietic and its leukopoietic properties.

[1] On sabbatical leave from Hadassah Medical Center, Hebrew University, Jerusalem, Israel.

Table II Radioactive tracer uptake in bones (day 10), mean right/left ratio ± 1 standard error

Assay		Group			
		I	II	III	IV
		irradiated			normal
		androgens, pre and post	androgens, post	untreated	
Femur	^{59}Fe	0.93±0.04	1.10±0.16	0.83±0.13	0.98±0.02
	^{99}Tc^m-coll	1.20±0.02	1.02±0.04	1.05±0.02	1.03±0.03
Tibia	^{59}Fe	0.81±0.04	0.88±0.14	0.49±0.11	1.05±0.03
	^{99}Tc^m-coll	1.49±0.39	1.27±0.12	1.30±0.08	0.95±0.10
Femur	^{59}Fe	0.88±0.04	0.98±0.12	0.62±0.12	1.01±0.00
and tibia	^{99}Tc^m-coll	1.28±0.13	1.10±0.05	1.13±0.04	0.99±0.05

increase in group 2 (36.9±6.2% on day 6, 90.5±34.4% on day 8) and only a slight increase in the control group 3 (28.4±4.1% on day 6 to 29.4±7.6% on day 8). The lymphocyte counts showed no change in group 2 over the controls on day 8, and group 1 showed only a slight elevation compared to the others.

Table II summarizes the right-to-left ratios in the femurs and tibiae obtained by isotopic techniques using ^{59}Fe and ^{99}Tc^m sulfur colloid. These results are summarized as mean right-to-left ratio ±1 standard error, stressing by this method the differences between the various groups. The ^{99}Tc^m-uptake ratio in the tibiae was higher in both testosterone treated groups (1 and 2) and in the irradiated controls (group 3) as compared with the unirradiated controls (group 4). No difference was found between groups 1 and 2. These differences with ^{99}Tc^m were not so pronounced in the femurs, in which only group 1 was found to be higher than the other 3 groups. On the other hand, the ^{59}Fe uptake ratio was higher in both the femurs and the tibiae in the groups receiving testosterone (groups 1 and 2) than in the irradiated controls (group 3). All results were more prominent in the tibiae than femurs. The combined results of femurs and tibiae are given in table II for both isotopes.

Discussion

The results demonstrate a definite relationship between testosterone propionate therapy and post irradiation leukopoietic recovery. Testoster-

Table 1. Absolute white blood cell and differential counts ± 1 standard error

Group											
irradiated									normal		
I: androgens, pre and post			II: androgens, post			III: untreated			IV		
WBC	P	L	WBC	P	L	WBC	P	L	WBC	P	L
17,380 ±800	3 040 ±460	14 010 ±500	–	–	–	–	–	–	–	–	
19,700 ±1,130	5,510 ±718	14,070 ±1,230	22,560 ±1,770	4,310 ±1,040	18,070 ±1,030	23,360 ±1,250	5,160 ±1,230	18,020 ±1,070	27,220 ±2,600	6,500 ±930	[illegible]
3,560 ±290	2,700 ±220	660 ±60	5,670 ±630	4,570 ±670	1,100 ±170	6,040 ±1,520	5,210 ±1,310	830 ±220	26,150 ±2,850	5,340 ±650	[illegible]
2,270 ±120	1,660 ±220	550 ±80	2,970 ±460	1,590 ±270	1,360 ±330	2,610 ±350	1,460 ±210	1,150 ±150	33,000 ±10,400	12,300 ±1,790	[illegible]
7,500 ±1,760	4,570 ±1,550	2,930 ±260	6,360 ±2,230	3,900 ±1,450	2,520 ±750	3,990 ±1,050	1,520 ±370	2,450 ±700	30,950 ±5,670	6,840 ±1,520	[illegible]

WBC = Absolute white blood cell count mm³, P = absolute polymorphonuclear leukocyte count m[illegible] absolute lymphocyte count mm³

during the first week of testosterone treatment in group 1 which is due mainly to a rise in the number of granulocytes ((from 3,040 ± 460 to 5,510 ± 718 in absolute numbers, or a rise from 100.0 ± 15.2 to 181.0 ± 23.5*.) The lymphocyte count during this period remained unchanged

Comparing the decrease in polymorphonuclear cells before radiation therapy (day 0) to the minimal count thereafter (day 6) showed that in group 1 the percent of cells decreased from 181.0 ± 23.5*. to 54.6 ± 6.9*. However, if compared to the starting point of the experiment in group 1 (day −7) the drop was from 100.0 ± 15.2*. to 54.6 ± 6.9*. This drop was less prominent in group 2 (100.0 ± 24.1*. on day 0, 36.9 ± 6.2*. on day 6), and even less in group 3 (100.0 ± 23.8*. on day 0, 28.4 ± 4.1*. on day 6)

The rise in white blood cells on day 8 of the experiment was compared to the minimal count on day 6. The change found was mainly due to differences in granulocyte numbers which showed a rise from 54.6 ± 6.9*. on day 6 to 150.1 ± 51.0*. on day 8, in group 1, a smaller

Unexpectedly, the $^{99}Tc^{m}$-sulfur colloid studies demonstrate greater tracer uptake in all 3 irradiated groups than in controls, with no differential effect evident from testosterone At 9 days postirradiation, one would expect little or no alteration of radiocolloid labeling from the studies of NELP *et al* [15], if anything slightly decreased uptake The increased labeling observed may well represent early hypervascularity and increased blood flow to the irradiated limb marrow, combined with normal efficacy of reticuloendothelial function and radiocolloid extraction, a recovery mechanism following irradiation (i e, response to injury) which is apparently not affected by androgens

In summary, the present results indicate a combined stimulation of the erythroid and the leukopoietic elements of the bone marrow by testosterone propionate Whether or not this dual stimulation is mediated via a common, possibly humoral, pathway cannot be determined, although our current knowledge of interrelationships between androgens and erythropoietin alone would lead one to suspect otherwise On the other hand, the multiple cell types of normal bone marrow customarily exist or travel together, and primary stimulation of erythroid recovery might lead to secondary early post irradiation recovery of the granulopoietic (and possibly also megakaryopoietic) marrow cells simply as a secondary cellular phenomenon, retaining normal cell interrelationships in the recovering marrow The known fact of a common marrow stem cell may be of fundamental importance in such a potential explanation However, the possibility of competition between leukopoiesis and erythropoiesis for a common stem cell has been considered in the rat [1, 2], and leukopoiesis was found to recover well after several oscillations in erythropoiesis had occurred

As with erythroid androgenic stimulation mediated via erythropoietin, it is possible that granulocytopoiesis may be mediated through an entirely separate leukopoietic factor (leukopoietin G, LIF) [3, 7, 16] which would act on the white blood cell precursors, and which might also respond to androgenic stimulation The present study has demonstrated enhancement of both leukopoiesis and erythropoiesis by testosterone propionate Another androgen ($7\beta,17\alpha$-dimethyltestosterone) used in breast cancer patients has been found to act specifically on platelets, less on white blood cells, and not at all on hematocrit [13] Thus, it may also be that different specific androgens exert their effects on different, probably committed precursors in the bone marrow at early stages of differentiation

one caused a greater rise of circulating polymorphonuclear leukocytes 8 days after X-irradiation in rats with one leg shielded than in controls. Additional testosterone given prophylactically before irradiation (group 1) caused a greater rise of the polymorphonuclear leukocytes at 8 days than did the hormone given only after irradiation (group 2). In a previous paper [10], it was postulated that the leukopoietic effect is dose-dependent. Therefore, the possibility exists that the differences in the rise of the white blood counts between group 1 and 2 might be caused by the differences in total duration of testosterone treatment between the 2 groups.

Testing of testosterone propionate as a possible leukopoietic agent before X-ray therapy (prophylactic) and after (therapeutic) [12] has shown that if the hormone was given before X-ray it stimulated the bone marrow, and the drop in white blood cells therafter was less prominent because of the already stimulated bone marrow. After X-ray therapy the prophylactic group rose less than the therapeutic group. However, the present study demonstrates that preirradiation treatment with testosterone can produce an augmented post-irradiation rise in circulating granulocytes. Possible explanations for these differences might lie in different X-ray dosages, which were lower in the previous studies (100–600 R) than in the present study (800 R), or in the shielding of one leg, which left significant volume of unirradiated bone marrow. This shielded marrow was previously stimulated by the androgen and, therefore, would be more active after X-ray at the time that the rest of the bone marrow was depressed. The results demonstrate a definite effect of testosterone propionate on stimulation of bone marrow when given prophylactically, causing a higher rise in white blood cells during recovery time than testosterone given only after X-ray treatment.

The marrow ^{59}Fe uptake studies again demonstrate the known stimulatory effect of testosterone on the erythroid marrow in addition to its leukocytic effect. In the groups treated with androgens both before and after X-ray, the ^{59}Fe uptake was significantly higher than in controls. This effect was more prominent in the tibiae, perhaps due in part to difficulties in total shielding of the proximal femurs, but perhaps also reflecting some greater ability of distal sites in the appendicular skeleton to respond to hematopoietic stimulation (a fact which reaches its extreme expression in adult man with total loss of functioning tibial marrow in a resting state).

Acta Haemat 48 307-311 (1972)

Acute Superficial Phlebitis in a Patient with Hemophilia A

Probably a Iatrogenic Effect

A Girolami and G Cella

University of Padua Medical School Institute of Semeiotica Medica, Padua

Abstract The occurrence of an acute superficial phlebitis in a patient with hemophilia A is reported The patient is a 29 year-old hemophiliac who was hospitalized because of a severe posttraumatic left knee hemarthrosis The phlebitic changes involved the left basilic vein and appeared after 9 days of hospital stay, while the knee condition had already considerably improved No changes in the underlying coagulation defect were present at the time of the onset of the phlebitis During the hospital stay, besides fresh or frozen plasma, the patient was given i v analgesic therapy namely Novalgin, a pyrazolon derivative, and Talwin, pentazocine lactate It is assumed that the phlebitic changes were secondary to the administration of the compounds The process disappeared in 3 days with no therapy

Key Words
Hemophilia
Phlebitis in hemophilia

The occurrence of thromboembolic disease or myocardial infarction in patients with congenital coagulation disorders represents an extraordinary event Thromboembolism was observed postoperatively or after a trauma in 2 patients with factor-VII deficiency [8] and in a patient with Hageman trait [15] Spontaneous popliteal vein thrombosis was also described in a patient with factor-V deficiency [14] Multiple pulmonary emboli were found at autopsy of 3 patients with congenital afibrinogenemia treated with fibrinogen [5, 13] Myocardial infarction was described in 3 patients with Hageman trait [10, 12, 17], in a patient with hemophilia B [4], and in 2 patients with hemophilia A [2, 3] Another patient with hemophilia A was found at autopsy to have widespread atherosclerosis [16] To our knowledge, no superficial or deep phlebitis or phlebothrombosis has ever been described in patients with either hemophilia A or B The purpose of this note is to describe the occurrence of an acute superficial phlebitis in a patient with hemophilia A

References

1 Baum, S. J. and Wyant, D. E.: Hematopoietic recovery in irradiated dogs. Radiat. Res. 44: 531–544 (1970)

2 Baum, S. J., Wyant, D. E., and Vagher, J. P.: Comparative hematopoietic cytokinetics in X-ray or gamma-neutron irradiated rats. Amer. J. Physiol. 216: 582–588 (1969)

3 Bierman, H. R., Marshall, G. J., Maekawa, T., and Kelly, K. H.: Granulocytic activity of human plasma. Acta haemat., Basel 27: 217–228 (1962)

4 DeGowin, R. L., Miller, S. H., and Grund, F. M.: Studies of recovery of erythropoiesis after irradiation as a function of the cellularity of the endosteum and spleen. J. Lab. clin. Med. 77: 219–227 (1971)

5 Dresch, C. et Najean, Y.: Cinétique des polynucléaires neutrophiles chez l'homme. Rev. europ. Ét. clin. biol. 15: 729–739 (1970)

6 Gordon, A. S., Handler, E. S., Siegel, C. D., Dornfest, B. S., and Lobue, J.: Plasma factors influencing release in rats. Ann. N.Y. Acad. Sci. 113: 766–789 (1964)

7 Gordon, A. S., Neri, R. O., Siegel, C. D., Dornfest, B. S., Handler, E. S., Lobue, J., and Eisler, M.: Evidence for a circulating leucocytosis inducing factor (LIF). Acta haemat., Basel 23: 323–341 (1960)

8 Hajdukovic, S.: The role of microenvironmental factors in hematologic response of mice exposed to 1000 r split dose irradiation and testosterone treatment. Strahlentherapie 141: 464–471 (1971)

9 Horn, Y.: A comparison of two androgenic hormones as leucopoietic agents in irradiated rats. Oncology 26: 16–24 (1972)

10 Horn, Y.: Leucopoietic properties of four different androgenic hormones in irradiated rats. Scand. J. Haemat. 8: 470–475 (1971)

11 Horn, Y.: The effect of androgenic hormones on bone marrow of rats receiving chemotherapy. Oncology 25: 512–520 (1971)

12 Horn, Y. and Robinson, E.: Testosterone as a bone marrow stimulant in irradiated rats. Radiol. clin. biol. 40: 133–141 (1971)

13 Horn, Y., Halden, A., and Gordon, G. S.: The platelet stimulating effects of 7β,17α-dimethyltestosterone. Blood (in press)

14 Horn, Y., Robinson, E., and Hochman, A.: Leukopoietic stimulation by testosterone in cancer patients treated by chemotherapy and irradiation. Radiol. clin. biol. 37: 29–35 (1968)

15 Neep, W. B., Mahendra, M. G., Larson, S. M., and Bower, R. E.: Long-term effects of local irradiation of the marrow on erythron and red cell function. Blood 36: 617–622 (1970)

16 Scott, W. P., Matthews, J. L., and Brady, K. L.: Effect of leukopenia and leukocytosis on tumor growth and survival time. Cancer 26: 124–137 (1970)

17 Schooley, M. and Benjamin, R. T.: Leukocytosis inducing factor in the blood of irradiated rats. Radiat. Res. 33: 82–93 (1968)

Authors' address: Dr. Yoav Horn and Dr. D. C. Price, Oncological Department, Hadassah Medical Center, POB 499, Jerusalem (Israel)

Acta Haemat 48 307-311 (1972)

Acute Superficial Phlebitis in a Patient with Hemophilia A

Probably a Iatrogenic Effect

A Girolami and G Cella

University of Padua Medical School Institute of Semeiotica Medica, Padua

Abstract The occurrence of an acute superficial phlebitis in a patient with hemophilia A is reported. The patient is a 29-year-old hemophiliac who was hospitalized because of a severe posttraumatic left knee hemarthrosis. The phlebitic changes involved the left basilic vein and appeared after 9 days of hospital stay while the knee condition had already considerably improved No changes in the underlying coagulation defect were present at the time of the onset of the phlebitis During the hospital stay, besides fresh or frozen plasma, the patient was given i.v analgesic therapy namely Novalgin, a pyrazolon derivative, and Talwin, pentazocine lactate It is assumed that the phlebitic changes were secondary to the administration of the compounds The process disappeared in 3 days with no therapy

Key Words
Hemophilia
Phlebitis in hemophilia

The occurrence of thromboembolic disease or myocardial infarction in patients with congenital coagulation disorders represents an extraordinary event. Thromboembolism was observed postoperatively or after a trauma in 2 patients with factor-VII deficiency [8] and in a patient with Hageman trait [15] Spontaneous popliteal vein thrombosis was also described in a patient with factor-V deficiency [14] Multiple pulmonary emboli were found at autopsy of 3 patients with congenital afibrinogenemia treated with fibrinogen [5, 13] Myocardial infarction was described in 3 patients with Hageman trait [10, 12, 17], in a patient with hemophilia B [4], and in 2 patients with hemophilia A [2, 3] Another patient with hemophilia A was found at autopsy to have widespread atherosclerosis [16] To our knowledge, no superficial or deep phlebitis or phlebothrombosis has ever been described in patients with either hemophilia A or B The purpose of this note is to describe the occurrence of an acute superficial phlebitis in a patient with hemophilia A

Case Report

The patient is a 22-year-old known hemophiliac who had been followed by us for the past 4 years as an outpatient. The hemorrhagic manifestations have been those typical of a patient with hemophilia of moderate severity. He had several hemarthroses in practically all joints but specially in the left knee. All hemarthroses were posttraumatic in nature, never spontaneous. Easy bruising has also been present during all his life. Epistaxes have been rare. On July 7, 1971 the patient slipped while working and hit his left knee. A severe hemarthrosis ensued in a few hours. His private physician attempted an aspiration of the joint which yielded after some manipulation a few milliliters of blood. Because of severe pain and because of the marked swelling of the joint with limitation of movement, the patient was admitted to our Department.

On admission, there was marked swelling of the left knee. There was no redness but the knee was tender. Limitation of movement was extreme. The patient was placed at absolute bed rest and 250 ml of fresh frozen plasma were given immediately. Because of the severe pain, Novalgin (a pyrazolon derivative) and Talwin (pentazocine lactate) i.v. were ordered. An oral semisynthetic 6-amino-penicillanic acid derivative (ampicillin) and an oral group B vitamin complex preparation were also prescribed, as we were suspicious that the attempted joint puncture might have caused a secondary infection. However, no constitutional signs of infection were present (WBC were normal, ESR was normal or slightly elevated, the patient was afebrile).

On the following day the patient's condition was unchanged and therapy was continued at the average of 200-250 ml of fresh lyophilized or fresh frozen plasma per day. After a few days, the knee was slightly improving but local pain and tension continued to be present.

The i.v. injections of Novalgin and/or Talwin were always performed into a vein of either arm, with no predilection of site, about every 5 h. It was subsequently calculated that the patient received, during the 9-day period, a total of 35 vials (18 Novalgin vials + 17 Talwin vials). Two days after the last plasma transfusion, while the left knee swelling had almost completely disappeared, the patient presented pain in the anteromedial aspect of the left arm area, radiating up to the axilla. Inspection of the area revealed the presence of a cord-like red area starting from the elbow and reaching the anterior aspect of the axilla. Novalgin and Talwin i.v. injections were discontinued and blood samples for a coagulation study were drawn. No significant change was observed from the pattern noted in the past (table I). In about 24 h, the phlebitic process subsided to disappear completely in 3 days with no therapy.

Afterwards, the patient's hospital stay was uneventful. The knee function was gradually regained and the patient could be discharged a few days later in good condition.

Material and Methods

The material used and the coagulation methods followed have been discussed in detail elsewhere [6, 7].

of Novalgin and/or Talwin. The possibility that the transfusional therapy might have contributed cannot be ruled out, but this does not seem likely. In fact, the last plasma transfusion was given 2 days before the onset of the phlebitic tenderness.

The i.v. administration of Novalgin is widely used by us for the relief of pain in cases of hemophilic hemarthrosis and we have never encountered difficulties. We had never used Talwin before in the treatment of hemarthrosis, but this compound is widely used as an analgesic and no secondary phlebitis has ever been observed [9]. It is likely that the combination of the 2 drugs might be responsible for an irritative action exercised on the venous wall. No definitive conclusions can be drawn from this observation. However, the observation in itself is exceptional and seems to emphasize once again the validity of VIRCHOW's observation that alteration of the venous wall may play an important role in the pathogenesis of thrombosis [1].

It could be safely assumed that the phlebitic process did not proceed in our patient to become a full-fledged thrombophlebitis because of the coagulation defect. Our observation seems to suggest also that the phlebitic changes of the venous wall may be independent of the intrinsic coagulation pattern.

References

1 ALEXANDER, B.: Blood coagulation and thrombotic diseases. Circulation 25: 872 (1962).
2 BORCHGREVINK, C. F.: Myocardial infarction in a hemophiliac. Lancet i: 1229 (1959).
3 BOIVIN, J. M.: Infarctus du myocarde chez un hémophile. Arch. Mal. Cœur 47: 351 (1954).
4 BRODY, J. I. and BEIZER, L. H.: Christmas disease and myocardial infarction. Arch. intern. Med. *115*: 552 (1965).
5 DE VRIES, A.; ROSENBERG, T.; KOCHWA, S., and BOSS, J. H.: Precipitating antifibrinogen antibody appearing after fibrinogen infusions in a patient with congenital afibrinogenemia. Amer. J. Med. 30: 486 (1961).
6 GIROLAMI, A.; MOLARO, G.; LAZZARIN, M.; SCARPA, R., and BRUNETTI, A.: A new congenital hemorrhagic condition due to the presence of an abnormal factor X (factor X Friuli). A study of a large kindred. Brit. J. Haemat. *19*: 179 (1970).
7 GIROLAMI, A.; STICCHI, A.; LAZZARIN, M., and SCARPA, R.: Congenital hypoprothrombinemia. Case report. Acta haemat., Basel *44*: 164 (1970).
8 GODAL, H. C.; MADSEN, K., and NISSEN-MEYER, R.: Thromboembolism in patients with total proconvertin (factor VII) deficiency. Acta med. scand. *171*: 325 (1962).

9 Goodman, L. S. and Gilman, A. (ed.): The pharmacological basis of therapeutics. 4th ed. (Macmillan, London 1970).
10 Glueck, H. I. and Roehll, W.: Myocardial infarction in a patient with a Hageman (factor XII) defect. Ann. intern. Med. 64: 390 (1966).
11 Hellem, A. J.: The adhesiveness of human blood platelets in vitro. Scand. J. clin. Lab. Invest. (suppl.) 51: 1 (1960).
12 Hoak, W. E., Swanson, L. W., Warner, E. D., and Connor, W. F.: Myocardial infarction associated with severe factor XII deficiency. Lancet ii: 884 (1966).
13 Ingram, G. I. C., Mc Bride, D. J., and Spencer, H.: Fatal pulmonary embolism in congenital fibrinopenia. Acta haemat., Basel 35: 56 (1966).
14 Miller, S. P.: Coagulation dynamics in factor V deficiency: a family study with a note on the occurence of thrombophlebitis. Thromb. Diath. haemorrh. 13: 500 (1965).
15 Ratnoff, O. D., Busse, R. J., and Sheon, R. P.: The demise of John Hageman. New Engl. J. Med. 279: 760 (1968).
16 Stewart, J. W. and Acheson, E. D.: Atherosclerosis in a hemophiliac. Lancet i: 1121 (1957).
17 Vroman, L.: personal communication.

Authors' address: Dr. A. Girolami and Dr. G. Cella, Istituto di Semeiotica Medica, Università di Padova, Via Ospedale Civile 43, *Padova* (Italy)

Acta Haemat. 48: 312–319 (1972)

Heteromorphic Pair of Metacentric Chromosomes with Fused Arms and the Philadelphia Chromosome in a Case of Acute Myeloid Leukemia[1]

M. H. KHAN[2]

Zentrum der Inneren Medizin der Johann Wolfgang Goethe Universität, Abteilung für Hämatologie (Direktor Prof. Dr. H. MARTIN), Frankfurt/Main

Abstract. The cytogenetic studies of a 61-year-old male with an acute myeloid leukemia revealed prior to antileukemic therapy a heteromorphic pair of chromosomes with fused arms and mid-located centromere. Also the Ph1 chromosome and loss of A3, B and G accompanied by gain of D and E members, were consistent karyotypic findings. The karyotypic pattern of different cell lines was suggestive of a common stem cell origin.

Key Words
Acute myeloid leukemia
Chromosome anomalies in leukemia
Karyotype
Marker chromosomes
Ph1 chromosome

Unlike the typical Ph1 chromosome of the chronic myeloid leukemia (CML), the aberrations in acute myeloid leukemia (AML) are generally characterized by some non-specific kind of aneuploidy or pseudodiploidy. There are only a few reports of morphologically abnormal marker chromosomes in AML. KISTOLOGII and ROBSON [4] reported in 2 cases of AML an extra-densely stained chromosome of D size with little arm repulsion. SANDBERG *et al.* [9] reported a marker ring chromosome in a patient of AML, who had been previously treated with 5-fluorouracil. Abnormal minute markers [9] and the Ph1 chromosomes have been reported in rare cases of AML [3, 5].

The purpose of this communication is to present a case of AML exhibiting a heteromorphic pair of chromosomes with fused arms and mid-located centromere. Also the Ph1 chromosome as well as extra chromo-

[1] Presented in part at the 13th International Congress of Hematology, Munich 1970.

[2] Supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg.

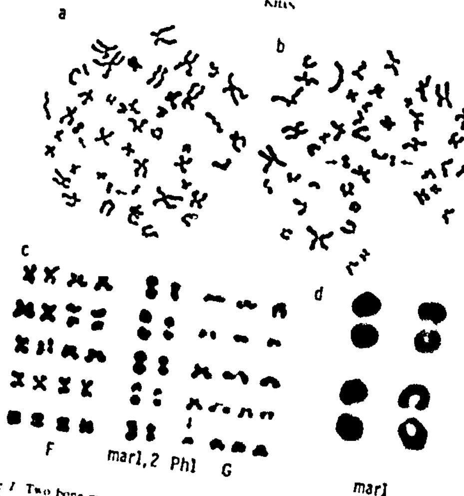


Fig 1 Two bone marrow mitoses with *a* 45 and *b* 46 chromosomes. Except the 2 markers with fused arms and mid located centromere (arrows) all others showed good arm repulsion. *c* Partial karyotypes of groups F and G and markers (mar1 mar2) from 5 cells (including the 2 metaphases of this figure). The Ph1 chromosome is present in the last row. *d* Enlarged mar1 selected from 4 different cells illustrating the morphology.

balled or rod shaped elements having tetra or near tetraploid number. The remaining 40% of the metaphase plates had a clear chromosomal morphology (fig 1 and 2). 40 metaphase plates with clear chromosomal morphology were examined for the presence of marker chromosomes and for the chromosomal number distribution. Predominant cell line was pseudodiploid (25%) followed by 45 (22%) and 47 (20%) cell lines (table 1). The absence of normal diploid cells in the bone marrow preparation was apparently due to the mitotic advantage of leukemic cells over the

Table I Cytogenetic anlaysis of bone marrow cells prior to therapy and peripheral blood cells after therapy

	41	42	43	44	45	46	47	48	53	89	Total	%
Bone marrow cells	–	1	3	3	9	10	8	4	1	1	40	
					22%	25%	20%					
mar1	–	1	3	2	9	10	8	4	1	–	38	95.0
mar2	–	1	3	3	8	10	8	4	1	–	38	95.0
Ph1	–	–	1	1	3	7	7	2	–	–	21	52.5
Blood cells with PHA	1	–	2	1	1	19	3	1	–	–	28	
after therapy						68%	11%					
mar1	1	–	1	1	–	1	2	1	–	–	7	25.0
mar2	1	–	1	1	–	1	2	1	–	–	7	25.0
Ph1	1	–	–	–	–	–	2	–	–	–	3	10.7

normal hematopoietic cells 95% of the cells exhibited a heteromorphic pair of chromosomes with unusual morphology being characterized by fused arms and mid located centromere Measurements of the surface area (with the help of parchment paper) of the markers (mar1, mar2) and F group chromosomes, revealed that except for 3 cells, in all other metaphases karyotyped, the mar1 was slightly larger than the chromosome 19 and the mar2 was a little smaller than the chromosome 20 32 bone marrow cells of excellent quality were karyotyped In addition to the presence of marker chromosomes (mar1, mar2, Ph1), loss of A3, B and G accompanied by gain of D and E (17 or 18) members were consistent findings Occasionally deletion of the short arm of a B (Bp-) member and a large D (Dq+) chromosome were also observed (table II, fig 1 and 2) 50 well spread mitoses of tetraploid category with contracted, balled and rod shaped elements were photographed and examined for their number distribution (table III) They showed an approximate duplication of the diploid category of cells having clear chromosomal morphology

The peripheral blood cultures were examined during the antileukemic therapy A total of 28 metaphases were investigated for the presence of marker chromosomes and the Ph1 chromosome 68% of the cells had normal karyotypic pattern, except for increased chromosomal breakage These cells were apparently PHA stimulated lymphocytes Marker chromosomes with fused arms were present in 25% of the cells observed, 11% showed also the Ph1 chromosome (table I)



Fig. 2. a Karyotype of a cell with 46 chromosomes, showing the markers (mar1, mar2) and the Ph1 chromosome. Loss of A, B and G members is associated by gain of one large D (arrow) and one E chromosome. Note the deletion of short arm of a B chromosome (arrow). *b* Karyotype of a cell with 47 chromosomes showing the same pattern as above, except for 2 extra D chromosomes instead of one extra D chromosome.

Table II Karyotype analyses of bone marrow cells

Chromosome No	Cells, karyotyped	Affected series A 3	B	C	D	E 17–18	G	Marker chromosomes Ph1	mar1	mar2	Additional findings[1]
43	1	2−	1−	−	1+	1+	4−	−	1	1	Dq+, ace (2×)
44	2	2−	1−	1−	1+	1+	2−	−	1	1	Dq+ (2×)
	1	2−	1−	−	1+	−	2−	1	−	1	Bp-, ace (1×)
45	6	2−	1−	−	1+	1+	2−	−	1	1	Dq+ (5×) Bp- (3×),
	2	2−	1−	1−	1+	1+	2−	1	1	1	ace (1×)
46	7	2−	1−	−	1+	1+	2−	1	1	1	Dq+ (7×), Bp- (3×) ace (1×)
	3	2−	1−	−	1+	1+	1−	−	1	1	Bp-
47	5	2−	1−	−	2+	1+	2−	1	1	1	Dq+ (1×)
	1	2−	1−	−	2+	1+	1−	−	1	1	Bp-
48	2	2−	1−	−	2+	1+	1−	1	1	1	Bp- (1×)
	2	2−	1−	−	2+	1+	−	−	1	1	Bp- (1×)
	Total 32										

[1] × = Mitosis, Bp = short arm deletion of B chromosome, Dq+ = enlarged D chromosome, ace = acentric

Table III Chromosomal number distribution in the degenerative type of bone marrow cells

	Chromosome No 88	89	90	91	92	93	94	95	96	Total cells
Number of cells	5	5	9 18%	−	13 26%	2	11 22%	3	2	50

Discussion

The close relationship of the karyotypes of pseudodiploid and near diploid sublines with marker chromosomes suggest that they have developed from a common stem cell by gradual neoplastic evolution Neoplastic tissue, unlike the normal tissue, tends to mitotic instability, producing new chromosomes, which though seem to be obstacle for smooth mitotic function of the cell carrying them, are nevertheless maintained for a long

period in tumor cell line [6]. Numerical changes and structural rearrangements are frequent components of clonal evolutions [2]. This may lead to random heterogenity which is regarded by WISCT [10] as the basis for a continous stem line selection during the further progressive development of an established malignant tissue. Even in heterogenous population stepwise karyotypic evolution may be deduced which may show a predominant cell or stem line [7]. Various structural and numerical aberrations in this case occurred before therapy which suggest that this might have been the quest for the adaptability to the tumor environment.

The two marker chromosomes of similar morphology but different sizes might have evolved from one common ancestral chromosome. Their peculiar form might have been the results of loss of the distal portion of arms and or translocation of material and subsequent fusion of the telomeric portions. It is also probable that one of them has developed from the other one as the consequence of some anaphase disturbance.

The karyotypic analyses suggest that deletions are distributed on different chromosomes and therefore it is quite uncertain from which chromosome these marker elements have evolved. Of course it is also possible that the two chromosomes have originated independently from different chromosomes and their common occurrence is merely fortuitous. The two metacentric marker chromosomes (mar1 mar2) had well defined morphology being characterized by fused arms in the form of two equal bits and unbroken circumference. One was slightly larger the other smaller than the chromosomes of the group F. In the densly stained elements a cleft from the centromere to the middle of the two equal bits was visible. In some instances two rings or crescent like structures communicating via centromere were apparent. The morphology of the markers (mar1 mar2) is illustrated in figures 1 and 2. In a case of benzene induced erythroleukemia a marker ring chromosome of some what similar configuration has been reported by FORNI and MOREO [1]. In contrast to the mar1 and mar2 all other chromosomes showed good arm repulsion. Another interesting finding in this case was the presence of the Ph1 defect in the bone marrow cells (52.5%) (fig. 1 and 2) and less frequently in the mixed population of PHA stimulated peripheral blood culture preparations (table I).

In this case the Ph1 chromosome occurred commonly in association with the two other markers. In spite of the chromosomal number variation their karyotypic patterns were consistent characterised by loss of A3, B and G accompanied by gain of D and F members as well as by the presence of the three conspicuous marker elements.

It seems justified to conclude that the cell lines around the pseudodiploid mode might have occurred through non-disjunction initiating the process of clonal evolution, while the mechanism of the degenerative supercontracted elements might have been impelled by some mitotic disturbance Sensitivity to colchicine could also be made responsible for this phenomenon Although normal chromosomal findings is not an evidence against malignancy, such kind of multiple aberrations suggest that the disease is in a highly malignant stage Their occurrence before the chemotherapy may signify the role of chromosomes in the cancerogenesis

Acknowledgement

I wish to acknowledge the expert technical assistance of Miss CHRISTIANE MECKLENBURG

References

1 FORNI A and MOREO L Chromosome studies in a case of benzene induced erythroleukaemia. Europ J Cancer *5* 459–463 (1969)

2 GROUCHY J DE and NAVA C DE, A chromosomal theory of carcinogenesis Ann intern. Med *69* 381–391 (1968)

3 KHAN M H und MARTIN, H Myeloblastenleukamie mit Philadelphia Chromosom Klin Wschr *16* 821–824 (1967)

4 KINLOUGH M A and ROBSON H N Study of chromosomes in human leukaemia by a direct method Brit. med. J *ii* 1052–1055 (1961)

5 KIOSSOGLOU K A MITUS W J, and DAMESHEK W Chromosomal aberrations in acute leukemia Blood *26* 610–641 (1965)

6 LEVAN A The significance of polyploidy for the evolution of mouse tumor Strains of the TA3 mammary adenocarcinoma with different ploidy Exp Cell Res *11* 613–629 (1956)

7 [illegible]

8 [illegible]

9 [illegible] and CROSSWHITE, L. H Chromo- [illegible] N Y Acad Sci *113* 663–716 (1964)

10 WINGE, Ö Zytologische Untersuchungen über die Natur mal gner Tumoren. II Tierkarzinome bei Mausen Z. Zellforsch mikr Anat. *10* 683–735 (1930)

Author's address Priv Doz Dr med D Sc M HUSSEIN KHAN, Zentrum der Inneren Medizin Abt fur Hamatologie Johann Wolfgang Goethe Universitat, Theodor Stern Kai 7 *Frankfurt/Main* (FRG)

I. Perlick, W. Plenert und O. Prokop: Fortschritte der Hämatologie. Zytomorphologie, Serologie, Immunologie, Hämostaseologie. vol. 1. Ausgewählte Abhandlungen über Physiologie und Pathophysiologie der Erythro- und Thrombozytopoese sowie der Protektine. Barth, Leipzig 1970. 611 pp., 134 fig., 101 tab., M 72.–.

In dieser auf 4 Bände vorgesehenen Reihe sollen Übersichtsreferate die Ergebnisse hämatologischer Grundlagenforschung für die klinische Anwendung vermitteln. Im Vordergrund dieses Bandes stehen biochemische und morphologische Arbeiten über die rote Blutzellreihe: Hämoglobinopathien (S. R. Hollán), medikamentös bedingte Störungen (J. Libánský), exogene Methämoglobinämien (H. Benaschik), Elektronenmikroskopie (H. Löwe), Membran (L. Lacko), Mitosestudien (E. G. Rondanelli, E. Magliulo), Erythroblastenkinetik (L. Bianchini), neurohumorale Regulationen (W. N. Tschernigowski *et al.* vom Pawlow-Institut Leningrad). A. Mäsky schildert die Thrombozytenimmunologie mit eingehenden methodischen Darstellungen. Die kurzen Beiträge von A. Morczek über Strahlenhämatologie und von G. Ruffey über Knochenmarkkulturen stehen etwas ausserhalb des gezogenen Rahmens. G. Uhlenbruck und G. I. Pardoe liefern (in englischer Sprache) einen Beitrag über die Immunchemie der Blutgruppensubstanzen. Als Protektine werden Agglutinine aus Schnecken und Fischrogen bezeichnet, die sowohl mit Erythrozyten wie auch mit Bakterien reagieren und als immunologische Schutzmechanismen für die abgelegten Eier dieser Tierarten aufgefasst werden. Über ihre Biochemie und ihre Reaktionen wird von O. Prokop, G. Uhlenbruck, W. Köhler und G. I. Pardoe berichtet.

Jedem Kapitel ist ein ausführliches Literaturverzeichnis beigegeben. Die Beiträge sind gut dokumentiert, inhaltlich zum kleineren Teil mehr propädeutisch, meist speziell referierend mit hohem wissenschaftlichem Niveau. Die Autoren und Herausgeber haben mit diesem gut ausgestatteten Band ein verdienstvolles Werk begonnen.

S. Witte, *Karlsruhe*

Editorial

25 Years of Acta Haematologica

Acta Haemat. 48: 322-330 (1972)

The Fibrinolytic Enzyme System in Leukaemia, Myelomatosis and Myeloproliferative Diseases

D. OGSTON, AUDREY A. DAWSON and HELEN M. ADAM

Departments of Medicine and Pathology, University of Aberdeen, Aberdeen

Abstract. Components of the fibrinolytic enzyme system were measured in 22 adult patients with leukaemia, 21 with myelomatosis and 10 with myeloproliferative disorders. Alterations compatible with disseminated intravascular coagulation (DIC) were found in half the patients with acute leukaemia and in some patients with myelomatosis. The changes of DIC did not appear to correlate with haemorrhagic features. The patients with idiopathic thrombocythaemia had low fibrinolytic activity.

Key Words
Fibrinolysis
Haemorrhagic diathesis
Intravascular coagulation
Leukaemia
Myelomatosis
Myeloproliferative disorders

A haemorrhagic tendency is a common feature in patients with leukaemia and may occur in other forms of haematological malignancy such as myelomatosis, and in idiopathic thrombocythaemia. Bleeding in leukaemia and myelomatosis is usually associated with thrombocytopenia, but cases in which the haemorrhagic tendency was ascribed to pathological fibrinolytic activity or disseminated intravascular coagulation (DIC) have been described. In this study, the major components of the fibrinolytic enzyme system have been assayed in adult patients with leukaemia, myelomatosis and myeloproliferative disease.

Material and Methods

Plasminogen activator was assessed by performing euglobulin clot lysis times by the method of Nilsson and Olow [24]. The results were expressed by plotting the lysis times logarithmically against units of fibrinolytic activity [34], 10 units being

arbitrarily equated with a lysis time of 50 min. Plasminogen activator was also measured by applying 30 μl samples of resuspended euglobulin precipitate to fibrin plates prepared from 0.2% human fibrinogen (Grade L, AB Kabi, Stockholm) After incubation at 37 °C for 24 h, the area of lysis was estimated from the product of 2 diameters at right angles to each other A partially purified preparation of urokinase (Leo Pharmaceutical Products, Ballerup, Denmark) was used as a reference standard The areas of lysis produced by the urokinase standard dilutions were plotted on a double logarithmic scale and the fibrinolytic activity of the euglobulin precipitate obtained by extrapolation and expressed as Ploug units of urokinase [29]

Plasminogen was measured by the caseinolytic technique of ALKJAERSIG *et al* [1] and the results expressed in Sherry units [2]

Fibrinogen was assayed by a modification [26] of the method of RATNOFF and MENZIE [31]

Serum inhibitor of plasminogen activation was measured using a standard clot system as described by BENNETT [3] One unit of inhibition is equivalent to that produced by 10^{-3} M epsilon aminocaproic acid

α_1 Antitrypsin and α_2-macroglobulin levels were assayed by single radial immunodiffusion [18] using reagents obtained from Hoechst Behringwerke AG

Serum fibrin degradation products (FDP) were measured by the tanned red cell haemagglutination inhibition technique of MERSKEY *et al* [20]

Platelet counts were performed visually using the method of OETTLE and SPRIGGS [25]

Control values were obtained from healthy volunteers and hospital patients convalescent from minor disorders which are not known to influence the fibrinolytic system

22 *leukaemic patients* were studied. The type of leukaemia was diagnosed by examination of peripheral blood and bone marrow aspirates using standard cytochemical stains where these were required.

The diagnosis of *myelomatosis* (21 patients) was made by serum protein analysis, bone marrow smear appearances and radiological appearances.

The myeloproliferative disorders (polycythaemia vera, chronic myeloid leukaemia and idiopathic thrombocythaemia) (10 patients) were identified by clinical features and haematological examination supplemented by radioisotopic estimation of the total red cell mass in the case of polycythaemia vera.

Results

The mean levels with standard deviations of the components of the fibrinolytic enzyme system found in control subjects are shown in table I.

Leukaemia Ten patients with chronic lymphatic leukaemia were studied The range of values with means are presented in table II. There were no striking deviations from the normal levels found in control subjects. In contrast, 5 of the 10 patients with acute leukaemia (table III) had elevated levels of FDP (over 20 μg/ml) The mean plasminogen of these 5 pa-

Table I. Components of the fibrinolytic enzyme system in control subjects

	Number	Mean age years	Mean	SD
Plasminogen activator, units	30	56.3	4.2	1.3
Plasminogen, casein u/ml	65	56.0	4.4	0.5
Fibrinogen, mg/100 ml	65	56.0	373	55
Serum activation inhibitor, units	20	57.4	3.5	1.0
Fibrin degradation products, µg/ml	20	56.1	6.5	4.0
α_1-Antitrypsin, mg/100 ml	50	57.3	251	50
α_2-Macroglobulin, mg/100 ml	50	57.3	271	45

tients was 3.4 units/ml compared with 4.7 units/ml for the 5 patients with FDP in the normal range. In addition, when the influence of age was taken into account, 3 of the 5 patients with elevated FDP had a reduced fibrinogen concentration. Fibrinolytic activity, as assessed by the euglobulin clot lysis times and by the area of lysis produced by euglobulin precipitate on a fibrin plate, was in the normal range in 8 patients; 2 had a reduced level. The combination of elevated FDP levels with reduced plasminogen concentration and absence of increased fibrinolytic activity suggests that some of the patients with acute leukaemia had some degree of DIC. It is notable, however, that haemorrhagic features correlated more closely with the presence of severe thrombocytopenia than with the laboratory features of DIC (table III). The serum inhibitor of plasminogen activation was raised (over 5.5 units) in 3 of the 10 patients with chronic lymphatic leukaemia and in 2 patients with acute leukaemia. α_1-antitrypsin levels were considerably increased in patients with acute leukaemia (mean 470 mg%). α_2-macroglobulin levels were moderately elevated (mean 363 mg%).

Myelomatosis. 21 patients with myelomatosis were studied. They showed a wide range of values for the components of the fibrinolytic system. The majority of plasminogen activator levels lay within the normal range. 5 patients had abnormally low levels (under 2 units) and a single patient had an increased level (20 units). A plasminogen level of under 3 casein units/ml was found in 4 of the 20 patients in whom it was measured. A moderate increase in the fibrinogen concentration was observed in many patients, but it exceeded 600 mg/100 ml in 2 patients only. Assay

ıble II Range and mean values for components of the fibrinolytic enzyme system in patients with leul myelomatosis and myeloproliferative disorders

	Number of patients	Mean age, years	Plasminogen activator units	Plasminogen casein u/ml	Fibrinogen mg/100 ml	Fibrin degradat products μg/ml
'hronic lymphatic ukaemia	10	70 3	3 5–12.4 (5 7)	2.7–5 1 (4 1)	220–740 (396)	2 3–16 0
'hronic myeloid ·ukaemia	2	52 3	5 8–6 6 (6 2)	4 1–4 3 (4 2)	245–335 (290)	13 0–52
cute leukaemia	10	65 2	1 0–9 0 (4 6)	2 9–5 6 (4 1)	190–980 (443)	4 1–89 6
1yelomatosis	21	69 5	1 0–20 0 (4.3)	2 3–6 3 (4 2)	260–750 (458)	46–128 0
olycythaemia vera	7	58 7	4.2–8.2 (6 4)	3 3–5 0 (3 8)	200–425 (287)	4 8–19 2
rimary ırombocythaemia	3	70 7	1 3–3 3 (2.1)	3 5–4 7 (4 3)	355–800 (512)	0–19 2 ('

Table III Components of the fibrinolytic enzyme system in patients with acute leukaemia

Subject No	Sex	Age, years	Type of leukaemia	Severity of haemorrhagic features	Platelet count × 1,000/μl	Plasminogen activator, units	Plasminogen casein u/ml	Fibrinogen mg/100 ml	Fibrin degradation products μg/ml
1	M	34	myeloblastic	+ + +	20	3 8	3 7	265	56 8
2	M	67	myeloblastic	+ + +	11	1 1	4 1	275	6 1
3	M	87	myeloblastic	+	28	9 0	4 3	460	11.2
4	M	64	stem cell	+	207	3 6	4 5	530	4 1
5	M	44	myelomonocytic	+ +	18	3 3	5 6	500	19 2
6	F	72	myelomonocytic	+ +	51	7 1	3 6	220	25 6
7	M	84	monoblastic	0	117	5 5	3 3	530	89 6
8	F	50	promyelocytic	+ +	36	7 1	2.9	190	35 2
9	M	71	monoblastic	+ + +	33	4 0	5 1	980	14 4
10	F	79	myelomonocytic	+ +	59	<1 0	3 5	480	51.2

of FDP was performed in 13 patients 7 had raised levels (over 20 μg/ml) and 2 of these had a level of over 100 μg/ml Neither of the 2 patients with a FDP level in excess of 100 μg/ml had haemorrhagic features One was notable in having sustained a pathological fracture 3 days before

study and in having a peripheral white count of 12,000/mm³, including 33% myeloma cells. She died the following day. The other patient died of a massive pulmonary embolus 2 days after study. It is likely that the increased FDP level resulted from deep venous thrombosis or an earlier pulmonary embolus rather than from DIC [33].

Assay of the serum inhibitor of plasminogen activation was performed in 11 patients with myelomatosis. Five patients had a decreased level (under 1.5 units) while a single patient had an increased level. A number of the patients studied had increased α_1-antitrypsin levels (mean 384 mg/100 ml). The α_2-macroglobulin levels (mean 263 mg/100 ml) were mainly within the normal range.

Myeloproliferative diseases. While no notable abnormality was found in the 7 patients with polycythaemia vera in respect of plasminogen activator, FDP and inhibitor levels, 5 of the patients had reduced levels of fibrinogen when the effect of age was taken into account, and the mean plasminogen level of the patients was reduced, compared with healthy controls.

The 2 patients with chronic myeloid leukaemia had slightly reduced fibrinogen levels. One patient had an elevated FDP level.

The 3 patients with primary thrombocythaemia (platelet count 970,000–1,200,000/mm³) had a decrease in plasminogen activator activity and the serum inhibitor of plasminogen activation was raised in the 2 patients in whom it was measured.

Discussion

This study was initiated to ascertain the frequency of changes in components of the fibrinolytic enzyme system in patients with haematological malignancies and myeloproliferative disease, and to assess the relationship, if any, of these changes to the haemorrhagic features found.

A number of reports have appeared on abnormal fibrinolysis in acute leukaemia [8, 9, 23–28]. Promyelocytic leukaemia was considered to be specific in respect of hypofibrinogenaemia [11, 13, 32], but it has been noted that reduced fibrinogen levels are not restricted to that form of acute leukaemia [14]. Disseminated intravascular coagulation has been suggested as a cause for the hypofibrinogenaemia [11, 14], and laboratory evidence of DIC was found in 4 patients with acute leukaemia by MERSKEY *et al.* [19]. The finding in this study of increased FDP levels, in the

absence of increased fibrinolytic activity, would support the view that DIC is not uncommon in acute leukaemia The contribution of such a degree of DIC to clinical features, however, is doubtful, severe DIC causing haemorrhagic features is probably rare in acute leukaemia

We have previously reported that reduced plasminogen levels are not infrequent in leukaemia [27] BRAKMAN *et al* [7] have also observed low plasminogen levels in some of their patients with acute leukaemia Causes other than DIC are presumably responsible for a proportion since the FDP levels were not elevated in all patients with a lowered plasminogen level

Increased fibrinolytic activity has been described in patients with myelomatosis [35] NILEHN and NILSSON [22] found that 30 of 57 patients had 'high' fibrinolytic activity and the serum of 8 of 19 patients had detectable fibrinolytic split products The 5 patients studied by MOHLER *et al* [21] however, showed no alteration in fibrinolytic activity or plasminogen concentration The present series of 21 patients had no consistent alterations in fibrinolytic components, but the finding of raised FDP in the serum of a proportion suggests that DIC may occur in myelomatosis with some frequency PRESTON and LEE [30] reported on a series of 12 patients with myelomatosis, 4 had evidence of DIC with a rise in the serum FDP level Abnormalities in fibrinogen fibrin conversion may also occur in myelomatosis [12] LACKNER *et al* [17] have noted inhibition of fibrin monomer gelation in 2 patients with a prolonged thrombin clotting time

A number of investigatore have reported increased fibrinolytic activity in patients with polycythaemia vera [5, 10], and low fibrinogen levels have also been described [4 10] BLOMBACK *et al* [6] noted that 3 of 6 patients studied with polycythaemia vera had significantly increased fibrinogen turnover rates and suggested that this resulted from increased consumption In the present study decreased fibrinogen levels have been found in some of the patients with polycythaemia vera but an increase in FDP was not found in these patients Accordingly we have obtained no evidence from this small series that DIC occurs in polycythaemia vera The cause of the reduced fibrinogen levels is obscure

In view of the presence of antiplasmin and anti activators in platelets [15 16 36] it is of interest that the patients with thrombocythaemia had low fibrinolytic activity None of these patients had the haemorrhagic tendency which is a common feature of idopathic thrombocythaemia

Our survey of patients with certain haematological malignancies and

myeloproliferative diseases indicate that significant alterations in components of the fibrinolytic system are infrequent in chronic lymphatic leukaemia and polycythaemia vera. Mild DIC, as manifested by an increase in FDP and a reduction in plasminogen or fibrinogen, appears to be relatively common in acute leukaemia and an occasional finding in myelomatosis. Its contribution to clinical features, however, appears to be minimal.

Acknowledgements. We are indebted to Mrs HELEN LEE and Mrs NAOMI HOOGSTADT for technical assistance, and to colleagues in the Aberdeen General Hospitals for permission to study their patients.

References

1 ALKJAERSIG, N., FLETCHER, A. P., and SHERRY, S.: The mechanism of clot dissolution by plasmin. J. clin. Invest. 38: 1086–1095 (1959).
2 ALKJAERSIG, N., FLETCHER, A. P., and SHERRY, S.: ε-aminocaproic acid: An inhibitor of plasminogen activation. J. biol. Chem. 234: 832–837 (1959).
3 BENNETT, N. B.: A method for the quantitative assay of inhibitor of plasminogen activation in human serum. Thromb. Diath. haemorrh. 17: 12–22 (1967).
4 BJORKMAN, S. E.: Three cases of polycythaemia with fibrinopenia. Acta med. scand. 129: 472–477 (1947).
5 BJORKMAN, S. E.; LAURELL, C. B., and NILSSON, I. M.: Serum proteins and fibrinolysis in polycythaemia vera. Scand. J. clin. Lab. Invest. 8: 304–308 (1956).
6 BLOMBÄCK, B.; CARLSON, L. A.; FRANZEN, S., and ZETTERQVIST, E.: Turnover of [illegible] labelled fibrinogen in man. Studies in normal subjects, in congenital coagulation factor deficiency states, in liver cirrhosis, in polycythaemia vera and in epidermolysis bullosa. Acta med. scand. 179: 557–574 (1966).
7 BRAKMAN, P.; SNYDER, J.; HENDERSON, E. S., and ASTRUP, T.: Blood coagulation and fibrinolysis in acute leukaemia. Brit. J. Haemat. 18: 135–145 (1970).
8 BROWN, R. C.; CAMPBELL, D. C., and THOMPSON, J. H.: Increased fibrinolysis with malignant disease. Arch. intern. Med. 109: 201–204 (1962).
9 COOPERBERG, A. A. and NEIMAN, G. M.: Fibrinogenopenia and fibrinolysis in acute myelogenous leukaemia. Ann. intern. Med. 42: 706–711 (1955).
10 DE VRIES, S. I.; PRAKT VAN STRAATEN, M. A. J.; MULLER, E., and WETTERMARK, M.: Antiplasmin deficiency in polycythaemia. A form of thrombopathy. Thromb. Diath. haemorrh. 6: 445–446 (1961).
11 DIDISHEIM, P.; TROMBOLD, J. S.; VANDERVOORT, R. L. E., and MIBASHAN, R. S.: Acute promyelocytic leukaemia with fibrinogen and factor V deficiencies. Blood 23: 717–728 (1964).
12 FRICK, P. G.: Inhibition of conversion of fibrinogen to fibrin by abnormal proteins in multiple myeloma. Amer. J. clin. Path. 25: 1263–1273 (1955).
13 [illegible], J. M. and BARKHAN, P.: Acute promyelocytic leukaemia. Postgrad. med. J. 42: [illegible] (196[illegible]).

14 Hirsch J, Buchanan J G, Gruchy, G C. de, and Baikie, A G Hypofibrinogenaemia without increased fibrinolysis in leukaemia Lancet i 418–420 (1967)
15 Johnson S A and Schneider, C. L. The existence of antifibrinolysin activity in platelets Science *117* 229–230 (1953)
16 Kwaan H C. and Suwanwela N Inhibitors of fibrinolysis in platelets in polycythaemia vera and thrombocytosis Brit J Haemat. *21* 313–322 (1971)
17 Lackner, H Hunt, V, Zucker, M B, and Pearson, J Abnormal fibrin ultrastructure polymerisation and clot retraction in multiple myeloma Brit J Haemat *18* 625–636 (1970)
18 Mancini G Carbonara, A O., and Heremans J F Immuno-chemical quantitation of antigens by single immunodiffusion Int J Immunochem *2* 235–254 (1965)
19 Merskey, C., Johnson, A J, Kleiner G J., and Wohl, H The defibrination syndrome Clinical features and laboratory diagnosis Brit J Haemat *13* 528–549 (1967)
20 Merskey C. Kleiner G J, and Johnson A J Quantitative estimation of split products of fibrinogen in human serum relation to diagnosis and treatment Blood *28* 1 18 (1966)
21 [illegible]

23 Nilsson I M Bjørkman S E. and Anderson, L. Clinical experience with Σ aminocaproic acid (Σ ACA) as an antifibrinolytic agent Acta med scand *170* 487–509 (1961)
24 Nilsson I M and Olow B Fibrinolysis induced by streptokinase in man Acta chir scand *123* 247–266 (1962)
25 Oettle A G and Spriggs E I New optical and cytochemical techniques in haematology in Dyke Recent advances in clinical pathology, 2nd ed (Churchill London 1951)
26 Ogston C. M and Ogston D Plasma fibrinogen and plasminogen levels in health and in ischaemic heart disease J clin Path. *19* 352–356 (1966)
27 Ogston D McAndrew, G M., and Ogston C. M Fibrinolysis in leukaemia J clin Path *21* 136–139 (1968)
28 Pisciotta A V and Schulz, E. J Fibrinolytic purpura in acute leukaemia. Amer J Med *19* 824–828 (1955)
29 Ploug J and Kjeldgaard N O Urokinase an activator of plasminogen from human urine I Isolation and properties Biochim biophys Acta *24* 278–282 (1957)
30 Preston F E and Lee D Fibrinolysis in myelomatosis. Acta haemat., Basel *47* 65–70 (1972)
31 Ratnoff O D and Menzie C. A new method for the determination of fibrinogen in small samples of plasma J Lab clin Med 37 316–320 (1951)
32 Rosenthal, R L. Acute promyelocytic leukaemia associated with hypofibrinogenaemia Blood *21* 495–505 (1963)
33 Ruckley C V, Das, P C. Leitch A G Donaldson A A Copland W A Redpath A T Scott P., and Cash J D Serum fibrin fibrinogen degradation

products associated with postoperative pulmonary embolus and venous thrombosis. Brit. med. J. *ii:* 395–398 (1970)
34 Sherry, S.; Lindemeyer, R. I.; Fletcher, A. P., and Alkjaersig, N.: Studies on enhanced fibrinolytic activity in man. J. clin. Invest. *38:* 810–822 (1959)
35 Sirridge, M. S.; Bowman, K. S., and Garber, P. E.: Fibrinolysis and changes in fibrinogen in multiple myeloma. Arch. intern. Med. *101:* 630–635 (1958)
36 Stefanini, M. and Murphy, I. S.: Studies on platelets. XIV. Human platelets as source of antifibrinolysin. J. clin. Invest. *35:* 355–361 (1956)

Authors' address: Dr. D. Ogston, Department of Medicine, Dr. A. Dawson and Dr. Helen M. Adam, Haematology Unit, Department of Pathology, University of Aberdeen, Foresterhill, *Aberdeen* (Great Britain)

Acta Haemat 48 331–336 (1972)

Sarcoidosis in a Patient with Cold Haemagglutinin Disease

E PALAZZO, F OBERLING, M-L NORTH, J-M LANG,
S MAYER and R. WAITZ

Clinique des Maladies du Sang and Centre de Transfusion Sanguine
(Chief Prof R. WAITZ) Strasbourg

Abstract A case of cold haemagglutinin disease treated with prednisone and immunosuppressive therapy, in which sarcoidosis developed is described The possible pathogenic relation between the two diseases is briefly discussed

Key Words
Cold haemagglutinins
Haemolytic anaemia
Sarcoidosis

The association of cold haemagglutinin disease (CHAD) and sarcoidosis has never, to our knowledge, been described CHAD has been considered a special form of macroglobulinaemia or a monoclonal disease [7] and some authors consider it as a lymphoproliferative disorder [20] TURKINGTON and BUCKLEY [22] described the association of macroglobulinaemia and sarcoidosis and they attached the macroglobulinaemia to an early manifestation of sarcoidosis The association of sarcoidosis with malignant lymphoma (Hodgkin's disease, lymphosarcoma reticulosarcoma) has seldom been described [2, 9] The possible transformation of sarcoidosis in lymphoma has been suggested [19] On the other hand, several cases of inmmunohaemolytic anaemia, none of the CHAD type, have been described in sarcoidosis [23]

Our paper reports a case of CHAD in which a sarcoidosis was diagnosed 3 years after her first admission

Case History

A 41 year-old woman was admitted to our clinic in February 1967 because during a blood donation a high titer of cold haemagglutinins had been found Since 1963 she was followed in another hospital for circulatory troubles (Raynaud s phe

products associated with postoperative pulmonary embolus and venous thrombosis. Brit. med. J. i: 395–398 (1970).

34 SHERRY, S.; LINDEMEYER, R. I.; FLETCHER, A. P., and ALKJAERSIG, N.: Studies on enhanced fibrinolytic activity in man. J. clin. Invest. 38: 810–822 (1959).

35 SIRRIDGE, M. S.; BOWMAN, K. S., and GARBER, P. L.: Fibrinolysis and changes in fibrinogen in multiple myeloma. Arch. intern. Med. 101: 630–635 (1958).

36 STEFANINI, M. and MURPHY, I. S.: Studies on platelets. XIV. Human platelets as source of antifibrinolysin. J. clin. Invest. 35: 355–361 (1956).

Authors' address: Dr. D. OGSTON, Department of Medicine, Dr. A. DAWSON and Dr. HELEN M. ADAM, Haematology Unit, Department of Pathology, University of Aberdeen, Foresterhill, *Aberdeen* (Great Britain)

She was readmitted in June 1970. The cure with chlorambucil had been followed only for 2 months, with no apparent beneficial results. In april 1970 she had presented an episode of acute bronchitis with jaundice. Physical examination showed the persistence of acrocyanosis and jaundice. Liver palpation was tender. The same immunohaematological and protein alterations were found. Conjugated bilirubin was elevated (1.4 mg/100 ml) and the Tc liver scan revealed a moderate hepatomegaly. The chest roentgenogram showed bilateral hilar adenopathies. A bronchial mucosa biopsy showed non-caseating epithelioid granulomas, suggestive of sarcoidosis (fig. 1). The Kveim test was positive. A test of lymphocytic transformation was performed. The results showed that the propositus lymphocytes underwent a poor stimulation in mixed culture and with PHA. She received no treatment.

The patient was readmitted in April 1971. Her cryopathic symptoms were unchanged. Direct bilirubin was normal. The sheep cells agglutination test was positive for the first time. Examination of the elution with the technique described by [illegible] and [illegible] in 19[illegible] [5] showed that the cold antibodies were of the IgM kappa type. The chest roentgenogram showed the disappearance of the hilar adenopathies and some parenchymal opacities on the right lung. The tuberculin skin test was negative.

Pertinent laboratory data are summarized in table I.

Discussion

Our patient fulfills the diagnostic criteria of CHAD: Raynaud's phenomenon, evidence of chronic haemolysis, a positive direct anti globulin test of the complement type and the presence in the serum of a high titred cold agglutinin [5].

Although the formation of sarcoid-like granulomas has been reported in different entities [1] and the presence of granulomas may result from many diseases [11], the presence in our patient of bilateral hilar adenopathy, the demonstration of non-caseating granulomas by bronchial biopsy [21] and the positive Kveim test are acceptable criteria for sarcoidosis [14]. Several possibilities may then be considered: (1) coincidental association, (2) the CHAD is an early manifestation of sarcoidosis, (3) the CHAD has favoured the appearance of sarcoidosis, (4) there is a common aetiologic agent or immunologic alteration that has favoured both diseases.

The first possibility is very difficult to discard particularly concerning entities of unknown causes and low frequency. As the Editorial of the British Medical Journal 1967 [3] points out 'statistics based on hospital studies are notoriously fallible when it comes to describing apparent association between diseases whose causes are unknown. The two-disease fal-

Fig. 1. June 1970. Biopsy of bronchial mucosa with noncaseating granuloma. H.E. × 170.

[illegible]meron) weight loss and fatigue. The patient had 2 sisters who suffered from rheumatoid arthritis. On examination she was found to be pale, slightly icteric and she showed acrocyanosis. She had no lymphoadenopathy nor hepatosplenomegaly. Her haemoglobin level was 10.7 g/100 ml, red cells 3,560,000/mm³, reticulocytes 7.6% and 7600 white cells per mm³ with a normal differential count. Serum bilirubin was 1.6 mg/100 ml, unconjugated type. Sternal bone marrow was hyperplastic with 24% of erythroblasts and 12.4% of normal plasmocytes. Serum total proteins were 8.4 g/100 ml and γ-globulins 2.26 g/100 ml. IgM was found increased by immunoelectrophoresis. Red cell survival studies with ⁵¹Cr labelled cells was shortened (13 days). Immunohaematological studies revealed a high titre (1/2048) of cold haemagglutinins with anti-I specificity. Acid autohaemolysins with bromelin treated red cells were present. The Coombs direct test was positive of the IgM and complement type. The tuberculin skin test was negative. All the investigations looking for a lymphoproliferative or autoimmune disease were negative. The patient was treated with [illegible] 10 months without beneficial results.

In February 1968 she was readmitted for severe [illegible]; physical examination was [illegible]. The laboratory revealed the persistence of the haemolytic anaemia and of the [illegible]. A tuberculin skin test was negative. A treatment with chloram[illegible] was [illegible].

She was readmitted in June 1970 The cure with chlorambucil had been followed only for 2 months, with no apparent beneficial results In april 1970 she had presented an episode of acute bronchitis with jaundice Physical examination showed the persistence of acrocyanosis and jaundice Liver palpation was tender The same immunohaematological and protein alterations were found. Conjugated bilirubin was elevated (1 8 mg/100 ml) and the Tc liver scan revealed a moderate hepatomegaly The chest roentgenogram showed bilateral hilar adenopathies A bronchial mucosa biopsy showed non-caseating epithelioid granulomas suggestive of sarcoidosis (fig 1) The Kveim test was positive A test of lymphocytic transformation was performed The results showed that the propositus lymphocytes underwent a poor stimulation in mixed culture and with PHA She received no treatment.

The patient was readmitted in April 1971 Her cryopathic symptoms were unchanged Direct bilirubin was normal The sheep-cells agglutination test was positive for the first time Examination of the elution with the technique described by GOLDBERG and BARNETT in 1967 [8] showed that the cold antibodies were of the IgM kappa type The chest roentgenogram showed the disappearance of the hilar adenopathies and some parenchymal opacities on the right lung The tuberculin skin test was negative

Pertinent laboratory data are summarized in table I

Discussion

Our patient fulfills the diagnostic criteria of CHAD Raynaud's phenomenon, evidence of chronic haemolysis, a positive direct anti globulin test of the complement type and the presence in the serum of a high titred cold agglutinin [5]

Although the formation of sarcoid like granulomas has been reported in different entities [1] and the presence of granulomas may result from many diseases [11], the presence in our patient of bilateral hilar adenopathy, the demonstration of non caseating granulomas by bronchial biopsy [21] and the positive Kveim test are acceptable criteria for sarcoidosis [14] Several possibilities may then be considered (1) coincidental association, (2) the CHAD is an early manifestation of sarcoidosis, (3) the CHAD has favoured the appearance of sarcoidosis, (4) there is a common aetiologic agent or immunologic alteration that has favoured both diseases

The first possibility is very difficult to discard particularly concerning entities of unknown causes and low frequency As the Editorial of the British Medical Journal, 1967 [3], points out 'statistics based on *hospital* studies are notoriously fallible when it comes to describing *apparent* association between diseases whose causes are unknown The two-disease fal

Table I. Laboratory data

	Feb 67	Feb 68	July 70	April 71
Hemoglobin g/100 ml	10.7	10.9	12.3 11.2	12.4
Reticulocytes %	7.6	1.0	1.0 5.2	2.6
Conjug. bilirubin mg/100 ml	0.0	0.0	4.3	0.1
Unconjug. bilirubin mg/100 ml	1.2	1.7	2.1	1.5
Bone marrow				
Erythroblasts %	24.8	36.4	20.4	37.6
Plasmocytes %	12.4	5.2	23.6	6.0
Lymphocytes %	6.8	8.8	10.0	7.2
Serum total proteins g/100 ml	8.4	7.6	7.2	7.5
γ-Globulins g/100 ml	2.2	1.8	1.8	1.9
Immunelectrophoresis	IgM↑ IgG IgA↓	IgM↑ IgA↓	IgM↑	IgM↑ IgA↓
Ig concentration mg/100 ml				IgA 103 IgM 833
Direct Coombs test	+	+	+	+
Erythrocyte coating substance	IgM C3		C3	C3
Cold agglutinin titre	1:2048	1:16000	1:4096	1:8192
Tuberculin skin test	–	–		–
Latex test				–
Sheep cell agglut.				1:128
Kveim test			+	

lacy creeps in – the interesting possessor of 2 diseases is far more likely to be written about than the humdrum patient with only one.

Concerning the second possibility, the elevation of serum globulins and the increased production of circulating antibodies has been classically described in sarcoidosis [4, 10, 18]. TURKINGTON and BUCKLEY [22] reported a case of macroglobulinemia with important cryopathic symptoms preceding by more than a year the diagnosis of sarcoidosis. This patient possessed in his serum a high molecular weight IgA and a cryoprecipitate composed of IgG and IgM globulins. The latex test was positive. The clinical evolution in our patient with CHAD symptoms preceding by several years the onset of sarcoidosis renders this second possibility improbable.

The third possibility, i.e. that the CHAD has favoured the appearance of sarcoidosis, could be explained by changes in the host immunity. The

CHAD is a type of paraproteinemia characterized by the production of autoantibodies (anti-Ii) Our patient presented also, as has been earlier reported in CHAD by COOPER and HOBBS [5], a deficit in IgA The tuberculin skin test was always negative The lymphocyte transformation test was performed after the onset of sarcoidosis The diminished transformation under PHA stimulation has been reported in sarcoidosis [13, 17] Lymphocyte transformation seems otherwise normal in CHAD [5] It is of interest to note that our patient and the patient described by TURKINGTON and BUCKLEY [22] had been treated by prednisone and chlorambucil some months prior to the diagnosis of sarcoidosis The changes induced by chemotherapy on the immune response [15] or the immunologic alterations in the host could have favoured the development of sarcoidosis by an unusual immunological reaction to phage infected bacteria [16], virus [12] pollen, etc.

Concerning the last possibility it is of interest to remark the presence of rheumatoid arthritis in 2 sisters of our patient One of them has been studied in our clinic and she showed a positive latex test, hypergammaglobulinemia with increased IgG, IgA and IgM levels and 7% of plasma cells in her bone marrow Her Gm and Inv phenotypes were identical with those of our patients This family constellation of immunity related entities could be an example of a genetic disorder, with immunologic imbalance leading to decreased cellular immunity and increased antibody response and autoantibodies formation [6]

Acknowledgements We are grateful to Dr LISE MARY ALVES DE LIMA who performed the elution of the cold antibodies

References

1 ANDERSON, R, JAMES D G, PETERS, P M, and THOMSON, A D Local sarcoid tissue reactions Lancet i 1211-1213 (1962)

2 ATWOOD W G, MILLER R C, and NELSON, C T Sarcoidosis and the malignant lymphoreticular diseases Arch Derm, Chicago 94 144-151 (1966)

3 Editorial Brit med J iv 568-569 (1967)

4 BUCKLEY C E, III and DORSEY, F C A comparison of serum immunoglobulin concentration in sarcoidosis and tuberculosis Ann intern Med 72 37-42 (1970)

5 COOPER, A G and HOBBS J R Immunoglobulins in chronic cold haemagglutinin disease Brit J Haemat 19 383-396 (1970)

6 Cummings N, Schall, G, Asofsky, R, Anderson, L, and Talal, N Sjögren syndrome Newer aspects of research, diagnosis and therapy Ann intern Med 75 937–950 (1971)

7 Gerbal, A, Homberg, J C, Rochant, H, Perron L et Salmon, Ch Les autoanticorps d'anémies hémolytiques acquises Nouv Rev franç Hémat 8 351–368 (1968)

8 Goldberg, L S and Barnett, I Mixed gG gM cold agglutinin J Immunol 99 803–804 (1967)

9 Goldfarb B L and Cohen, S S Coexistent disseminated sarcoidosis and Hodgkin's disease J amer med Ass 211 1525–1528 (1970)

10 Greenberger, G, Fizzi, T, Geraint, J D, and Bird R Serum proteins in sarcoidosis Lancet ii 1313–1315 (1964)

11 Guckian, J C and Perry, J L Granulomatous hepatitis An analysis of 63 cases and review of the literature Ann intern Med 65 1081–1100 (1966)

12 Hirshaut, Y, Glade, P, Vieira L O, Ainbender, E, Dvorak, B, and Siltzbach L Sarcoidosis, another disease associated with serologic evidence for herpes like virus infection New Engl J Med 283 502–506 (1970)

13 Hirschhorn K, Schreibman, R R, Bach F H, and Siltzbach, L E In vitro studies of lymphocytes from patients with sarcoidosis and lymphoproliferative diseases Lancet ii 842–843 (1964)

14 Israel, H L The diagnosis of sarcoidosis Ann intern Med 68 1323–1326 (1968)

15 Levin R H, Landy, M, and Frei E The effect of 6-mercaptopurine on immune response in man New Engl J Med 271 16 22 (1964)

16 Mankiewicz, E The relationship of sarcoidosis to anonymous bacteria Acta med scand, Suppl 425 68–73 (1964)

17 Mankiewicz, E, Kurti V, and Beland J Diagnostic procedures in sarcoidosis Canad med Ass J 104 684 690 (1971)

18 Norberg R Studies in sarcoidosis IV Serum immunoglobulin levels Acta med scand 181 497–504 (1967)

19 Rabin A C Bogdanovich, N K, and Golochevskaya, V S A case of transformation of sarcoidosis into reticulosarcomatosis Probl Hemat Blood transfusion 6 763 769 (1961)

20 Schubothe H The cold hemagglutinin disease Sem Hemat 3 27–47 (1966)

21 Siltzbach L E and Cahn L R Random biopsy of bronchial and palatal mucosa in the diagnosis of sarcoidosis Acta med scand Suppl 425 230 231 (1964)

22 Terrington R W and Buckley C E Macrocryoglobulinemia and sarcoidosis Amer J Med 42 156–164 (1967)

23 Weiss S und Mahr C Hämolytische Anämie bei Sarkoidose der Milz Acta haemat 37 126–136 (1967)

Author's address Dr J Oehling, Clinique des Maladies du Sang C H U F-67 Strasbourg (France)

present paper is to provide further details for an overall picture of this system during the pre- and post-natal life of the mouse.

Material and Methods

The techniques were already described in details [1]. Only the points at variance with what reported previously will be given here.

Animals. The mice used in the present experimental series were F_1 hybrids between inbred ♂ C3H and ♀ C57Bl, bred in our laboratory.

Age of embryos. In an attempt to increase the precision of the estimates by synchronizing the ovulation, hormonal treatment was applied to virgin female mice of 2–2.5 months [3]. About 60 h before mating the animals were injected i.p. with 2 IU of serum gonadotropin followed by a second injection of 2 IU of placental gonadotropin at about 10 h before mating. Three females were mated overnight with one male and scored the next morning for the presence of vaginal plugs. The age of embryos was determined taking as time zero the hour at which vaginal plugs were first seen.

Measurements of total red blood cells (RBC) in fetal mice. The estimates were based on the following technique of isotope dilution. Pregnant females were injected i.v. with about 5 μCi of ^{59}Fe in 0.2 ml of saline (ferric chloride, specific activity 3 mCi/mg) at 11 days after mating and sacrificed from the 14th day on. After sacrifice the embryos were excised and washed in cold saline while still enclosed in the amniotic sac. The membranes were then opened leaving the fetus attached by the umbilical cord. After a second careful washing and blotting the embryos were placed in heparinized plastic vials of known weight and containing a known volume of Hank's solution. The fetuses were dropped into the vials holding the membranes with small forceps and cutting with a pair of scissors the distal end of the umbilical cord. The radioactivity of the vials was immediately counted in a well-type scintillation spectrometer. The fetuses were then removed from the vial and the residual activity due to the haemorrhage blood in the remaining fluid counted again. Finally the number of red blood cells (RBC) per ml of suspension was estimated by standard haemocytometer counting after appropriate dilution. The total number of RBC in the fetus was obtained by the formula:

$$\text{total RBC/embryo} = \text{total RBC in vial} \times \frac{\text{activity of whole embryo}}{\text{activity of RBC suspension}}$$

Preparation of spleen and blood samples for colony forming unit (CFU) estimation. After excision the spleens were immersed in calcium free Hank's fluid supplemented with [illegible] newborn calf serum. Well dispersed suspensions of spleen cells [illegible] obtained from the age of 15.5 days of pregnancy by repeated aspiration [illegible] spleens in [illegible] of decreasing [illegible]. After [illegible] and in single cell [illegible] be obtained by [illegible] [illegible] [illegible] of a [illegible] [illegible] samples [illegible] [illegible] heparinized glass 7.5 [illegible] [illegible] the amount of blood obtained per

mouse was about 0.3 ml. Blood sam[illegible] [illegible]d, counted by Bürker haemocytometer and injected into recipient[illegible] [illegible] quots of whole blood. Spleen cell suspensions and fetal blood [illegible] [illegible]ed for their CFU content by the spleen colony technique [2] [illegible] [illegible]-rad irradiated animals, as described previously [1]. In the context of the present paper the number of CFU in blood samples is always r[illegible]ferred to the number of *nucleated* cells.

Experimen[illegible] pro[illegible]dures. Two experimental series were carried out on fetal mice to measure th[illegible] total number of RBC, involving estimates on approximately 2[illegible] fetuses at each [illegible]. Since the differences between the two series were extremely small and [illegible], the data are often presented as averages at each time with common [illegible]dard e[illegible]rs. In some instances, as in the estimates of the total number of sp[illegible] cells, the experiments were not done on single fetuses, but on pools of organ[illegible] [illegible] various an[illegible]mals. For this reason, statistical errors are not available.

Some of the est[illegible] [illegible] reported for adult animals were obtained as parts of different experiments [illegible] [illegible] comparable techniques.

Results

Changes in b[illegible] weight and cell number. Table I shows a summary [illegible] data concerning the body weight of fetal and newborn mice at various [illegible], the total number of spleen cells, the total number of circulating [illegible] and white blood cells (WBC). Other data on adult mice are also

[illegible] I. Changes [illegible]n body weight, number [illegible] spleen cells and number of circulating RBC [illegible] (standard errors of experimental estimates shown where possible)

[illegible]	Body weight g	Total number of nucleated spleen cells	Total number of circulating RBC	Total number of circulating WBC
			3.14±0.56×10[7]	–
[illegible] after vag[illegible]	0.344±0.013	9.67×10[4]	1.01±0.87×10[8]	–
[illegible] after vaginal [illegible]	[illegible]±0.016	5.31×10[5]	2.37±0.11×10[8]	1.58±0.31×10[5]
[illegible] after vaginal [illegible]	[illegible]±0.071	8.65×10[5]	2.94±0.14×10[8]	3.88±0.52×10[5]
[illegible] after vag[illegible]	1.0[illegible]±0.074	1.35×10[6]	3.97±0.21×10[8]	2.82±0.47×10[5]
[illegible] after vag[illegible]	[illegible]±0.051	3.73×10[6]	4.16±0.45×10[9]	6.19±1.19×10[5]
[illegible] after vag[illegible]	[illegible]±0.113	4.95×10[7]	–	–
[illegible] after birth	[illegible]619±0.145	1.15×10[8]	–	–
[illegible] after birth	23–2[illegible]	3.65×10[8]	1.81×10[10]	–
[illegible] after birth		2.09–		

included for comparison For what regards the spleen cells, their increase is continuous throughout fetal age and after birth, up to the adult life On a 'per gram' basis the increase in the number of cells covers a factor of about 10 during the intrauterine life and another factor of approximately 5 from birth to the adult age

The number of RBC is also continuously increasing during fetal life, roughly in parallel with the weight gain, so that in the course of this period the number of erythrocytes per gram of body weight remains constant between 3 and 4 · 10^9 RBC/g Compared with the fetus, however, the adult mouse has approximately twice as many RBC/g An analysis of the ratio RBC/WBC per ml of fetal blood showed no systematic changes between different groups of mice and remarkably constant average values between 1 and 2 × 10^3 during intrauterine life It may not be arbitrary, therefore, to attempt an estimate of the total number of circulating WBC based on the number of RBC/mouse Such estimates are given in the last column of table I and they may probably be considered representative of the number of WBC actually present in the blood stream irrespective of any compartmentalization of the whole WBC population whose presence in the fetus may be possible but was not checked These estimates, however, are certainly representative of the blood CFU estimates to be described later Between day 16 and 19 after vaginal plug the total number of circulating WBC is seen to increase about 4 times

CFU ratio of fetal spleen and blood cells By repeated experiments on spleen cell suspensions obtained from embryos of different ages and injected into heavily irradiated recipient mice, it was possible to estimate the number of CFU present at each fetal age per 10^5 nucleated spleen cells The relevant data are summarized in table II, and show an increase in cloning efficiency throughout the intrauterine life, followed by a sudden drop after birth At later times the CFU ratio becomes rather stable and in adult animals the values usually found for spleen cells are between 0.5 and 2.5 · 10^{-4} that is between 7 and 34 times less than the maximum value in the fetus

Cloning experiments were also carried out on fetal blood but their number is too limited to draw any definite conclusion about possible trends It is clear however that the cloning efficiency of nucleated blood cells decreases in the adult compared with the fetal age in agreement with what is seen in the spleen The weighted average CFU value per 10^6 nucleated circulating blood cells is taken to be 0.256 ± 0.023

Table II The average number of CFU/10^4 nucleated cells in mouse spleen cell suspensions and in the circulating blood (standard errors of experimental estimates shown where possible)

Age of the animals	Number of CFU/10^4 nucleated spleen cells	Spleens counted	Number of CFU/10^5 nucleated blood cells	Spleens counted
15 days after vaginal plug	0.413 ± 0.091	37	–	
16 days after vaginal plug	0.588 ± 0.053	92	–	
17 days after vaginal plug	1.186 ± 0.065	103	0.268 ± 0.030	12
18 days after vaginal plug	1.103 ± 0.061	79	0.250 ± 0.032	21
19 days after vaginal plug	1.696 ± 0.122	68	–	
1 week after birth	0.688 ± 0.064	47	–	
2 weeks after birth	0.352 ± 0.035	29	–	
3–4 months after birth	0.053 ± 0.250	500	0.068 ± 0.003	220

The number of CFU in the spleen and the blood stream Table III summarizes the estimates of the total number of CFU found on average in the spleen and in the circulating blood of the mouse These estimates were obtained by multiplication of the total number of nucleated cells and their CFU ratio at the various ages tested. In the case of the spleen no direct estimates of total CFU were attempted, as reported previously for the fetal liver [1] Since both the cell counting error and the variability of the CFU ratio affect the final estimates of table III, they should only be regarded as indications, useful for the purpose of comparing trends in the appearance and development of CFC populations in the organs tested Within these qualifications, the total number of spleen CFU is shown to increase steadily from the age of 15 days after vaginal plug up to 1 week after birth, and then to level off in the adult (table III) The number of about 5 000 CFU reported for the adult spleen should be taken as the best estimate that may be offered having regard to the extreme variability of the measurements between 1,000 and 9,000 CFU in apparently normal and uniform adult mice

Table III shows also that the total number of CFU in the blood stream changes with fetal age in relation to the increase of total nucleated cells In the adult, owing to the reduced cloning efficiency of the nucleated cells in the blood, the number of circulating CFU may be estimated at about 100

Table III. Estimates of the total number of CFU in the spleen and in the blood stream (errors on the experimental estimates given in preceding tables)

Age of the animals	Number of nucleated spleen cells	CFU/10^4 spleen cells	Number of WBC	CFU/10^4 nucleated blood cells	Total CFU spleen	Total CFU in blood
15 days after vaginal plug	9.67×10^4	0.413	–	–	4	–
16 days after vaginal plug	5.31×10^5	0.558	1.59×10^5	0.256	31	4
17 days after vaginal plug	8.65×10^5	1.186	3.88×10^5	0.256	103	10
18 days after vaginal plug	1.35×10^6	1.103	2.82×10^6	0.256	149	[illegible]
19 days after vaginal plug	3.73×10^6	1.696	6.19×10^6	0.256	633	16
1 week after birth	4.95×10^7	0.688	–	–	3 400	–
2 weeks after birth	1.15×10^8	0.352	–	–	4 048	–
3–4 months after birth	2.00–3.35×10^8	0.053–0.250	1.4×10^7 *	0.0[illegible]	5 000	95

* In a 25-gram animal of this strain the blood volume is ~2 ml, the number of nucleated blood cells is 7×10^6/ml.

A semi-quantitative outline of pre- and post-natal haemopoiesis in the mouse. On the basis of data reported in the present and the preceding paper [1], figure 1 is an attempt to summarize the kinetics of appearance, development and fate of haemopoietic CFC in the liver, spleen and blood of mice at various times before and after birth. It shows that CFC appear in the liver prior to 12 days of intrauterine life. From then on they grow at a progressively slower rate until the time of birth. Soon after then, a rapid disappearance takes place down to extremely low CFU values in the adult, in parallel with the cessation of haemopoietic activity of the liver (see also table II of preceding paper [1]). This pattern is qualitatively analogous for 2 different strains of mice, an outbred Swiss strain and the F_1 hybrid (C3H × C57Bl), and is confirmed by a direct technique of titration in the hybrid mouse.

Spleen colony formers increase rapidly in fetal life, starting from about 15 days, with an approximately exponential rate and a doubling time of about 14 h. After birth they do not disappear, but continue to increase at a slower rate and may be found in the adult in numbers of a few thousands. Blood CFU also increase during fetal life and remain in

the adult, so that a normal mouse has about 100 CFU circulating in the blood stream

Attempts to perform similar experiments on fetal bone marrow have failed owing to the technical difficulties of sampling this tissue prior to birth Therefore, the semi-quantitative picture of figure 1 may not at present be completed with the estimate of the bone marrow function which is the major haemopoietic tissue in adult life

Discussion

It is well known that in the adult mouse the curve of appearance of ^{59}Fe in blood cells reaches a plateau at about 72 h after injection of the label [4] A 3-day interval is in fact the most generally accepted in ^{59}Fe labelling experiments since the existence of a plateau is taken to mean that the transit of labelled RBC precursors from the blood forming tissues to the peripheral blood has been completed On this basis, collection of blood from fetal mice was started 72 h after iron labelling of the pregnant mothers In the mouse liver erythropoiesis begins at approximately 11 days of pregnancy and our own data have shown previously that in F_1 hybrids (C3H×C57Bl) it is well under way at 12 days [1] Since yolk-sac erythroid cells are still present in the circulating blood of C57Bl mice up to 14 days of pregnancy, in the proportion of 25–40% of total red cells [5] we may expect that the labelled cells are partly of yolk-sac and partly of liver origin The disappearance of yolk-sac cell after 14 days might probably contribute to some degree of iron reutilization in the fetus, a condition which is unfavourable for the precision of the dilution technique used

An analysis of ^{59}Fe radioactivity incorporated per fetus from 14 to 19 days of pregnancy has excluded in the present experimental series the presence of significant changes over this time lapse, so that the fetal mouse may be considered with respect to ^{59}Fe turnover, a reasonably closed system in the sense that there is no continous passage of labelled iron from the mother During the same time, however, the specific activity of erythrocytes (counts per 10^9 RBC) decreases by approximately a factor of 10 owing to the fact that cells labelled soon after iron injection are gradually diluted by a new population of unlabelled erythrocytes The precision of our estimates based essentially on this phenomenon, may have been affected by iron reutilization and/or storage, but the

Table III. Estimates of the total number of CFU in the spleen and [illegible] ferred on the experimental estimates given in previous [illegible]

Age of the animals	Number of nucleated spleen cells	CFU 10^4 spleen cells	Number of WBC	CFU 10^4 [illegible]
15 days after vaginal plug	9.67×10^4	0.413		
16 days after vaginal plug	5.31×10^5	0.[illegible]5	$1.[illegible] \times 10^5$	–
17 days after vaginal plug	8.65×10^5	1.186	3.85×10^5	[illegible]
18 days after vaginal plug	1.35×10^6	1.103	2.82×10	
19 days after vaginal plug	3.71×10^6	1.1[illegible]	6.19×10^5	
1 week after birth	4.95×10	0.6[illegible]	–	
2 weeks after birth	1.15×10^8	0.352	–	
3–4 months after birth	2.0[illegible]	0.053	1.4×10 *	
	7.35×10^8	0.250		

* In a 25-g animal of this strain the blood vo[lume] of blood cells is 7×10^6 [illegible]

A semi-quantitative outline of pr[illegible] the mouse. On the basis of data rep[illegible] ing paper [1], figure 1 is an attem[illegible] pearance, development and fa[illegible] spleen and blood of mice at [illegible] shows that CFC appear [illegible] From then on they g[illegible] birth. Soon after the[illegible] tremely low CFU val[illegible] haemopoietic activity [illegible] [1]). This pattern [illegible] mice, an outbred S[illegible] confirmed by a d[illegible]

Spleen co[illegible] about 15 day[illegible] time of ab[illegible] increase a[illegible] ca. th[illegible]

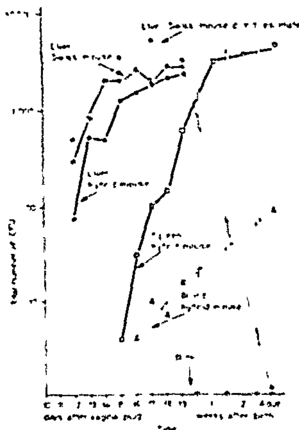


Fig. 1. A semi-quantitative estimation of the total number of CFU in the liver, spleen and peripheral blood and of the mouse at various times before and after birth.

erable scatter of their values. It is possible, however, to subscribe to the general conclusion drawn by these authors [12 and 13] that the concentration of CFU per ml of blood falls considerably after birth.

As to the significance of the changes observed with respect to the current theories of haemopoiesis, it appears difficult to draw definite conclusions in the absence of further knowledge about the flow rates of CFU in and from the various compartments tested. The present data are compatible with the view that migration of haemopoietic progenitor cells between different haemopoietic sites takes place via the blood stream

but do not exclude the autochthonous origin of the stem cells in each organ. It may be, however, significant that the loss of CFU concentration in the blood of the newborn and adult mouse, in comparison with the fetus, marks the cessation of the transient fetal and embryonic types of erythropoiesis and their complex changes from one to another organ and corresponds to the establishment of the stable adult bone-marrow erythropoiesis.

References

1 SILINI, G.; POZZI, L. V., and POMA, S.: Studies of the haemopoietic stem cells of mouse foetal liver. J. Embryol. exp. Morph. 17: 303–318 (1967).
2 TILL, J. E. and MCCULLOCH, E. A.: A direct measurement of the radiation sensitivity of normal bone-marrow cells. Radiat. Res. 14: 213–222 (1961).
3 SOUTHARD, J. L.; WOLFE, H. G., and RUSSELL, E. S.: Artificial insemination of dystrophic mice with mixtures of spermatozoa. Nature 208: 1126–1127 (1965).
4 BRIGANTI, G.; COVELLI, V.; SILINI, G., and SRIVASTAVA, P. N.: The distribution of erythropoietic bone marrow in the mouse. Acta haemat., Basel 44: 355–361 (1970).
5 MARKS, P. A.; FANTONI, A., and CHAPELLE, A. DE LA: Haemoglobin synthesis and differentiation of erythroid cells. Vitamins Hormones 26: 331–355 (1968).
6 FANTONI, A.: personal commun. (1972).
7 SIMINOVITCH, L.; MCCULLOCH, E. A., and TILL, J. E.: The distribution of colony forming cells among spleen colonies. J. cell. comp. Physiol. 62: 327–336 (1963).
8 POZZI, L. V. and SILINI, G.: Kinetics of multiplication and differentiation of haemopoietic progenitor cells transplanted into irradiated mice. In Effects of radiation on cellular proliferation and differentiation. IAEA, Vienna 1968.
9 MATIOLI, G.; VOGEL, H., and NIEWISCH, H.: The dilution factor of intravenously injected haemopoietic stem-cells. J. cell. comp. Physiol. 72: 229–234 (1968).
10 SMITH, W. W.; WILSON, S. M., and FRED, S. S.: Kinetics of stem cell depletion and proliferation. Effects of vinblastine and vincristine in irradiated mice. J. nat. Cancer Inst. 40: 847.
11 LAHIRI, S. K. and VAN PUTTEN, L. M.: Distribution and multiplication of colony forming units from bone marrow and spleen after injection in irradiated mice. Cell Tissue Kin. 2: 21–28 (1969).
12 BARKER, J. E.: Embryonic mouse peripheral blood colony forming units. Nature 228: 1305–1306 (1970).
13 BARNES, D. W. H. and LOUTIT, J. F.: Haemopoietic stem cells in the peripheral blood. Lancet ii: 1138–1141 (1967).

Authors' address: Dr. L. V. POZZI, Dr. U. ANDREOZZI and Dr. G. SILINI, Laboratorio di Radiobiologia Animale, CSN Casaccia, I-00060 S. Maria di Galeria (Roma) (Italy)

Acta Haemat 48 347–353 (1972)

The Effects of Hypertransfusion on the Formation of Zones of Iron Concentration in the Spleen of the Mouse Radiation Chimera

D Brynmor Thomas, Moyra E Marshall and A C. Riches

Department of Anatomy, The Medical School, Birmingham

Abstract The formation of zones of iron concentration in the spleen of the mouse radiation chimera is inhibited by transfusion induced polycythaemia This is consistent with the view that these zones represent colonies which contain appreciable numbers of erythroblasts On the surface of the spleen such colonies outnumber colonies which do not accumulate iron by more than 4 to 1.

Key Words
Bone marrow transplantation
Hypertransfusion in mice
Iron metabolism
Polycythaemia
Radiation chimera
Spleen iron

Macro-autoradiography has revealed the concentration of intravenously administered radioactive iron (^{59}Fe) in clearly defined zones in the spleen of the mouse radiation chimera [9] Eighty-five percent of these zones of iron concentration correspond in position to macroscopic surface colonies, surface colonies which thus appear to concentrate iron outnumber colonies which do not appear to concentrate iron, by more than 4 to 1 [8] Iron-concentrating colonies presumably include colonies composed of erythroblasts, together with mixed colonies which contain appreciable numbers of erythroblasts, while non-concentrating colonies presumably include mixed colonies which contain insignificant numbers of erythroblasts, together with colonies composed of granulocytes or megakaryocytes [8]

The relationship between the ability of a colony to concentrate iron and the varieties of cells which it contains cannot, however, be established directly, as the spleen is unsuitable for critical histological examination following compression during autoradiography, histological exam-

Table I The weight of the spleen, the number of macroscopic colonies visible on the surface of the spleen and the number of zones of iron concentration revealed in autoradiograms of the spleen, 9 days after exposure of mice to 750 rad of whole body X irradiation. Mean values and standard errors

Bone-marrow therapy	Hyper-transfusion	Number of animals	Spleen weight, g	Colonies	Zones
–	–	11	22.8 ± 2.2	1.2 ± 0.5	1.2 ± 0.3
+	–	7	39.1 ± 5.6	8.3 ± 1.9	5.6 ± 1.2
+	+	17	25.3 ± 1.2	2.6 ± 0.5	1.2 ± 0.3

Results (Table I)

The mean number of macroscopic colonies visible on the surface of the spleen 9 days after the administration of a lethal dose of whole body X-irradiation and the intravenous injection of 10^5 bone marrow cells is 8.3 ± 1.9 in non polycythaemic mice and 2.6 ± 0.5 in mice rendered polycythaemic by hypertransfusion on the day prior to irradiation ($p < 0.001$). Hypertransfusion thus results in a 69% reduction in the number of macroscopic colonies seen on the surface of the spleen.

Zones of iron concentration have been demonstrated in the spleen in both non polycythaemic mice (5.6 ± 1.2) and in hypertransfused mice (1.2 ± 0.3) ($p < 0.001$). Hypertransfusion has effected a 79 percent decrease in the number of zones observed and also a pronounced decrease in the size of the zones (fig. 2).

In irradiated animals which received neither bone marrow cells nor blood transfusions, 1.2 ± 0.5 macroscopic colonies were observed on the surface of the spleen and 1.2 ± 0.3 zones of iron concentration were revealed in autoradiograms of the spleen. The donated bone marrow cell suspension has thus been shown to contain 7.1 colony forming units (CFU) and 4.4 iron-concentrating colony forming units (FeCFU) per 10^5 cells. CFU and FeCFU distributed to extra splenic sites are not, of course, taken into account. Subject to certain reservations [4] it is possible to do so [6].

The weight of the spleen in non polycythaemic mice is significantly greater than in irradiated controls ($p < 0.01$). In polycythaemic mice, however, the weight of the spleen is not significantly different from the

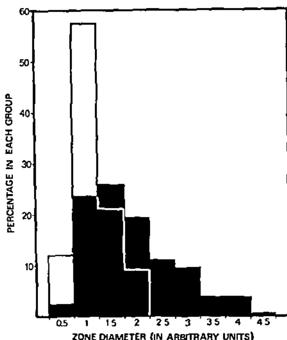


Fig 2 The effect of polycythaemia, induced by hypertransfusion, on the size distribution of zones of iron concentration in the spleen of the mouse radiation chimera □ Polycythaemic, ■ control

weight of the spleen in irradiated controls (p=0 3) and the spleen is significantly smaller than in non polycythaemic mice (p<0 01)

Discussion

Ten days after the treatment of lethally irradiated mice by the intravenous injection of haematopoietic cell suspensions, between 39 and 55% of the haematopoietic cells in the spleen are erythroblasts [7] The difference between the weight of the spleen in non-polycythaemic mice and in hypertransfused mice is not, therefore, surprising and is presumably due to inhibition of erythroblast production, following hypertransfusion [3] It thus appears to reflect the suitability of the regime which has

been employed during the present investigation to render animals polycythaemic. The suitability of this regime is endorsed by the decrease which it effects in the number of macroscopic colonies visible on the surface of the spleen. The 69 percent decrease which has been observed is similar in magnitude to the decrease (63%) observed by CURRY *et al* [1] and is more pronounced than the decrease (51%) reported by FELDMAN *et al* [2].

Although the formation of zones of iron concentration has not been completely inhibited by hypertransfusion, a 79 percent decrease in the number of zones has been observed together with a profound decrease in the size of the zones which are formed. Formation of such zones is thus highly susceptible to inhibition by transfusion induced polycythaemia. The suggestion that the zones represent colonies which contain appreciable numbers of erythroblasts [8] is therefore confirmed.

It has previously been demonstrated that on the surface of the spleen iron-concentrating colonies outnumber colonies which do not concentrate iron by more than 4 to 1. If the formation of all iron-concentrating colonies was prevented by hypertransfusion, a decrease in excess of 80% might thus have been expected in the number of colonies observed in polycythaemic animals, provided that no increase occurred in the number of non-concentrating colonies. As the formation of only 79% of the zones is inhibited, a decrease in excess of 63% in the number of surface colonies would have been predicted if it had been assumed that the proportion of superficially placed iron-concentrating colonies inhibited by hypertransfusion is not likely to be appreciably different from the proportion of all iron-concentrating colonies so affected. This predicted decrease would probably be an underestimate however because the decrease in zone formation is likely to be underestimated as the proportion of zones obscured by confluence or coincidence [8] is presumably greater in non polycythaemic mice than in hypertransfused mice since the zones are larger and more numerous in non polycythaemic mice. Conversely, the observed decrease will probably be exaggerated because very small erythroid colonies, which are relatively more numerous following hypertransfusion, are likely to escape recognition even though they may still be revealed in autoradiograms or histological preparations. The predicted decrease (≥63%) thus approximates well to the observed decrease (69%) and so provides confirmation for the previously estimated ratio of iron-concentrating to non-concentrating colonies on the surface of the spleen (4:1) [5].

Acknowledgements This investigation has been supported by a grant from the Medical Research Council It is a pleasure to acknowledge the help of Mr D J F Steers, Mrs C V Briscoe, Mrs. V Littlewood, Miss M Howdle, who prepared the line drawings, and Miss Anne Greenfield, who prepared the manuscript.

References

1 Curry, J L; Trentin, J J, and Wolf, N Hemopoietic spleen colony studies II Erythropoiesis. J exp Med. *125* 703–719 (1967)

2 Feldman, M, Bleiberg, I, and Liron, M Regulation of intrasplenic formation of erythroid clones. Ann N Y Acad Sci *129* 864–871

3 Jacobson, L O, Goldwasser, E, and Gurney, C W Transfusion induced polycythaemia as a model for studying factors influencing erythropoiesis, in Wolstenholme and O'Connor Ciba Found Symp Haemopoiesis, pp 423–445 (Churchill, London 1960)

4 Kretchmar, A L and Conover, W R Colony forming cells in the spleen Transplantation 8 576–581 (1969)

5 Schooley, J C Studies on the regulation of erythropoiesis in spleen colonies Exp Hemat. *7* 79 (1964)

6 Siminovitch, L, McCulloch, E A, and Till, J E The distribution of colony forming cells among spleen colonies. J cell comp Physiol *62* 327–336 (1963)

7 Thomas, D B The differentiation of transplanted haematopoietic cells derived from bone marrow, spleen and fetal liver J Anat, Lond. *110* 297–306 (1971)

8 Thomas, D B The relationship between zones of iron concentration and macroscopic surface colonies in the spleen of the mouse radiation chimera. J Anat, Lond (in press)

9 Thomas, D B, Steers, D J F, and Riches, A C A macro-autoradiographic method for the demonstration of iron-concentrating spleen colonies. Transplantation *10* 274–275 (1970)

10 Till, J E and McCulloch, E A A direct measurement of the radiation sensitivity of normal mouse bone marrow cells Radiat Res *14* 213–222 (1961)

Authors' addresses Dr D Brynmor Thomas and Dr A C Riches, Department of Anatomy, The Medical School, *Birmingham B15 2TJ* (England) Miss Moyra E. Marshall, The University of Rhodesia, PO Box MP167, *Mount Pleasant, Salisbury* (Rhodesia)

Acta Haemat. 48: 354–359 (1972)

Iron Release in the Reticuloendothelial Cells of the Spleen after Red Cell Damage

B. Iyengar and K. Chandra

Department of Pathology, Maulana Azad Medical College,
University of Delhi, New Delhi

Abstract. Equivalent hemolysis was produced after 24 h, in adult albino rats, by the intracardiac injection of copper sulphate, phenylhydrazine and antiserum. The erythrophagocytic count was done on sections of the spleen and compared with the release of Perls positive iron within the splenic reticuloendothelial cells (REC). From the results obtained it is concluded that iron is released within the splenic REC after erythrophagocytosis while the hemoglobin split directly into the plasma is not processed by these cells. The activity of the REC varies with the agent, copper sulphate giving rise to a rapid release of iron whereas phenylhydrazine and antiserum produce a slower activity.

Key Words
Erythrophagocytosis
Haemoglobin iron
Haemolysis
Splenic iron

Hemolysis can be extravascular or intravascular [1]. Erythrophagocytosis by the reticuloendothelial cells (REC) was studied after the production of acute hemolysis with different agents [2] as an index of extravascular hemolysis. After intravascular hemolysis the red cell iron is released into the plasma [3]. In both cases the red cell iron is split off from the hemoglobin molecule by the REC [4]. The present study was conducted to correlate the stainable iron released within the REC of the spleen after extravascular hemolysis with erythrophagocytosis as compared to intravascular hemolysis, releasing hemoglobin into the circulation.

Materials and Methods

Adult albino rats of both sexes weighing from 150 to 250 g, fed on stock laboratory diet and water *ad lib*, were used in all experiments. Copper sulphate (5,000 µg/200 g body wt), phenylhydrazine (20 mg/200 g body wt), and antiserum

ythrocyte serum (antiserum 1 ml of 1/10 dil/200 g body wt.) were injected by the intracardiac route to produce equivalent hemolysis after 24 h Batches of 9 rats each were sacrificed at intervals of 24, 48 and 72 h, 5 and 7 days. Serum bilirubin levels were estimated by the micromethod [5] immediately before injecting the hemolytic agent and before sacrificing the animal On post mortem the spleen was removed and fixed in buffered 10% formalin Paraffin sections, 5 μm in thickness were prepared. An erythrophagocytic count (EPC) was done as described in a previous study [2] on sections stained with hematoxylin and eosin. Sections from the same blocks were stained with Perl s stain for iron and the REC positive for iron were counted in a 100 high power fields (HPF) The average number of cells containing iron at each time interval was calculated A control group of 10 rats were sacrificed without producing hemolysis. The sections of the spleen were stained with Perl s stained and a count of the iron positive cells was done

Results

Copper Sulphate (fig 1a)

Serum bilirubin The highest level of serum bilirubin was observed 24 h after the injection of copper sulphate (3 1 mg%) It fell to 1 8 mg% on the 2nd day A mild secondary rise to 2 2 mg% was noted on the 3rd day Thereafter, the level gradually fell on the 5th and 7th day to 2 mg% and 1 9 mg%, respectively

Erythrophagocytosis The EPC was 27/HPF after 24 h The level gradually fell from 22/HPF on the 2nd day to 11/HPF on the 3rd day and to 9/HPF on the 5th day No activity was noted on the 7th day

Iron release A large number of REC show iron within their cytoplasm throughout the period of study The level rises to 78/HPF after 24 h and to a peak 96/HPF after 48 h Thereafter the level falls gradually to 69/HPF after 72 h, 52/HPF on the 5th day and 41/HPF on the 7th day

Phenylhydrazine (fig 1b)

Serum bilirubin After phenylhydrazine, the serum bilirubin level was above normal throughout. The level was 2 9 mg% after 24 h, and it further rose to 3 44 mg% after 48 h, 4 09 mg% after 72 h On the 5th day the level was maintained at this level, after which it fell to 3 31 mg% on the 7th day

Erythrophagocytosis The spleen showed a continuous erythrophagocytosis The rate was 17/HPF after 24 h, after which there was a sudden rise to 38/HPF after 48 h The level then gradually fell first to 31/HPF on the 3rd day, 30/HPF on the 5th day and 17/HPF on the 7th day

Iron release The iron level at first rises gradually to reach a peak after

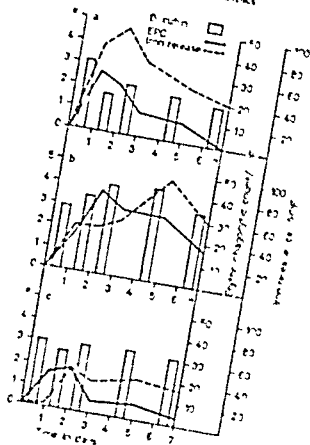


Fig 1 Curves showing the splenic erythrophagocytosis and the iron release within the RES. *a* After copper sulphate. *b* after phenylhydrazine, *c* after antiserum.

5 days. The level on the 1st and 2nd days are the same that is 41/HPF. The level rises to 51 HPF after the 3rd day and then to 94/HPF on the 5th day. There is a fall to 60 HPF on the 7th day.

Antiserum (Fig. 1c)

Serum bilirubin. Two peaks of bilirubin were observed after 24 h (3.15 mg%) and after 5 days (3.21 mg%). The level fell to 2.74 mg% on the 2nd day. The level was 3.07 mg% on the 3rd day and a slight fall was observed on the 7th day (3.19). Thus there was a sustained hemolysis throughout with slight fluctuations.

Erythrophagocytosis. Compared to the bilirubin levels the EPC in the spleen was low, the counts being 17 HPF after 24 h, 19 HPF after 48 h,

63 HPF after 72 h, 53 HPF after 5 days and 43 HPF after 7 days. Here there were two low peaks of erythrophagocytic activity. The pattern of EPC indicates a small component of extravascular hemolysis and a large component of intravascular hemolysis [2].

Iron release. The iron release in the RFC is relatively low as compared to the other two agents. There are two low peaks after 2 days (40 HPF) and 5 days (39 HPF). The first day showed a very slight rise to 31 HPF. After 72 h the level is 33 HPF and after 7 days it is 34 HPF.

The control animals showed no erythrophagocytic activity and no iron in the RFC of the spleen.

Discussion

The RFC is considered to be the chief cell process on effete red cells and hemoglobin [4]. Thus it was thought that equivalent hemolysis extravascular or intravascular should give rise to a similar release of iron within these cells. With this in view the iron release in the RFC was studied. The staining of the RFC gives an approximate quantitation. Perls positivity is seen when the ferritin iron is near saturation and in hemosiderin [6]. This iron is later released into the plasma for utilization in erythropoiesis [7].

From the above experiments it is seen that the pattern and the amount of iron release within the RFC varies with the hemolytic agent used, and it is evident that erythrophagocytosis plays an important role in the intracellular release of iron.

The pattern of iron release varies with the pattern of hemolysis. Copper sulphate produces a sharp hemolytic peak after 24 h which is mainly extravascular in nature as seen by the EPC peak [2] (Fig. 1a). Here the iron released rises rapidly and reaches a higher peak after 48 h and falls to a low level by the 7th day indicating that the release of iron from the red cell by the RFC is rapid.

Phenylhydrazine produces a progressive hemolysis [8], the splenic EPC having a high level after 48 h and then gradually falling (Fig. 1b). The peak EPC is seen on the 2nd day followed by a lower plateau extending to the 5th day before falling. The iron levels rise to a plateau extending to the 3rd day and reach a high peak on the 5th day, so that the iron levels rise slowly but reach a high level in the later half of the experiments. Here the release of iron is slow, the release occurring 3 days after

the peak EPC. The REC release the red cell iron slowly in spite of the high EPC. The iron release is prominent although it is slow.

Antiserum is the agent which produces a low splenic EPC as compared to the sustained high hemolysis (fig 1c) indicating that there is a prominent component of intravascular hemolysis [9]. Here the iron levels are low, the levels being maintained at this level with minor fluctuations. As the EPC is low and there is a prominent component of intravascular hemolysis, it indicates that the hemoglobin released during intravascular hemolysis has not been processed by the REC of the spleen. Thus the iron released in the splenic REC is closely linked with the erythrophagocytic activity with variations due to the agent used.

From the foregoing it is evident that the iron release in the splenic REC is closely linked with their [illegible] hemol[illegible] a h[illegible] [illegible]avascular has [illegible] produce [illegible] although equivalent hemolysis [illegible]. This indicates that the splenic REC do not appear to play an important role in processing the iron released during intravascular hemolysis. They process the red cell hemoglobin only after erythrophagocytosis of altered red cells.

It is also seen that agents producing varied red cell damage cause different patterns of REC activity in releasing iron. Copper sulphate gives rise to a rapid release of iron whereas phenylhydrazine gives rise to a slow release. Antiserum, on the other hand shows a low prolonged release. These features can be due to the interaction of the altered red cells and the REC.

Copper sulphate produces hemolysis by forming a coating of metalloprotein complex on the surface and by small agglutinates formed due to the multivalency of copper ion [10], giving rise to the EPC pattern [2]. Phenylhydrazine causes progressive red cell damage by altering the red cell membrane [8, 11]. Antiserum produces agglutination and intravascular breakdown of the red cells [12, 13]. The present experiments indicate that the breakdown of the red cells by the splenic REC is more rapid with the metalloprotein complexes and small agglutinates whereas damage to the red cell membrane or agglutinates with antiserum in some way inhibits the rapid release of iron.

Acknowledgement. We would like to express our grateful thanks to the Dean/Principal of the Maulana Azad Medical College, New Delhi, for giving us permission to publish this work.

References

1 Fairley, N. H.: Fate of extracorpuscular circulating haemoglobin. Brit. med. J. ii: 213-217 (1940).

2 [illegible], B. and Gupta, D. N.: Erythrophagocytosis as index of extravascular haemolysis (sent for publication).

3 Wintrobe, M. M.: Clinical Hematology (Lea & Febiger, Philadelphia 1962).

4 Noyes, W. D.; Bothwell, T. H., and Finch, C. A.: The role of the reticulo-endothelial cell in iron metabolism. Brit. J. Haemat. 6: 43-55 (1960).

5 King, E. J. and Wootton, I. D. P.: Micro-analysis in medical biochemistry, 4th ed. (Churchill, London 1964).

6 Saltman, P. and [illegible], A.: Mechanisms of iron storage; in Iron metabolism, A Ciba Symposium, p. 121 (Springer, Berlin 1964).

7 Lipschitz, D. A.; Simon, M. O.; Lynch, S. R.; Dugard, J.; Bothwell, T. H., and Charlton, R. W.: Some factors affecting the release of iron from the reticuloendothelial cells. Brit. J. Haemat. 21: 289-303 (1971).

8 [illegible], J. C.: Glutathione concentration and stability in the red blood cells in various disease states, and some observations on the mechanism of action of acetylphenylhydrazine. Brit. J. Haemat. 10: 437-444 (1964).

9 Jandl, J. H.; Jones, A. R., and Castle, W. B.: The destruction of red cells by antibodies in man. I. Observations on the sequestration and lysis of red cells altered by immune mechanisms. J. clin. Invest. 36: 1428-1459 (1957).

10 Jandl, J. H. and Simmons, R. L.: The agglutination and sensitization of red cells by metallic cations. Interactions between multivalent metals and the red cell membrane. Brit. J. Haemat. 3: 19 (1957).

11 Pisciotta, C. M., jr.: Drug induced blood dyscrasias. Disease-a-Month 1-52 (Oct. 1963).

12 Dacie, J. V.: The haemolytic anaemias. II. Autoimmune haemolytic anaemia (Churchill, London 1962).

13 Watterson, C.: The destruction of red blood corpuscles in experimental haemolytic anaemia. Acta med. scand. Suppl. 255 (1951).

14 Gross, F. (ed.): Iron metabolism, A Ciba Symposium (Springer, Berlin 1964).

Authors' address: Dr. [illegible] [illegible] and Dr. Kamla Chawla, [illegible], Pandara Road, New Delhi 3 (India)

the peak EPC. The REC release the red cell iron slowly in spite of the high EPC. The iron release is prominent although it is slow

Antiserum is the agent which produces a low splenic EPC as compared to the sustained high hemolysis (fig 1c) indicating that there is a prominent component of intravascular hemolysis [9] Here the iron levels are low, the levels being maintained at this level with minor fluctuations As the EPC is low and there is a prominent component of intravascular hemolysis it indicates that the hemoglobin released during intravascular hemolysis has not been processed by the REC of the spleen Thus the iron released in the splenic REC is closely linked with the erythrophagocytic activity with variations due to the agent used

From the foregoing it is evident that the iron release in the splenic REC is closely linked with their erythrophagocytic activity Intravascular hemolysis with the hemoglobin released into the plasma does not produce a high iron release within the splenic REC although equivalent hemolysis has been produced This indicates that the splenic REC do not appear to play an important role in processing the iron released during intravascular hemolysis They process the red cell hemoglobin only after erythrophagocytosis of altered red cells

It is also seen that agents producing varied red cell damage cause different patterns of REC activity in releasing iron Copper sulphate gives rise to a rapid release of iron whereas phenylhydrazine gives rise to a slow release Antiserum on the other hand shows a low prolonged release These features can be due to the interaction of the altered red cells and the REC.

Copper sulphate produces hemolysis by forming a coating of metalloprotein complex on the surface and by small agglutinates formed due to the multivalency of copper ion [10] giving rise to the EPC pattern [2] Phenylhydrazine causes progressive red cell damage by altering the red cell membrane [5 11] Antiserum produces agglutination and intravascular breakdown of the red cells [12 13] The present experiments indicate that the breakdown of the red cells by the splenic REC is more rapid with the metalloprotein complexes and small agglutinates whereas damage to the red cell membrane or agglutinates with antiserum in some way inhibits the rapid release of iron

Acknowledgements We would like to express our grateful thanks to the Director Principal of the Maulana Azad Medical College New Delhi for giving us permission to publish this work.

T. Travnicek and J. Neuwirt (eds.): **The Regulation of Erythropoiesis and Haemoglobin Synthesis.** Universita Karlova, Praha 1971. 428 pp.

This book contains reports on the proceedings of the International Symposium organized from August 10th to 13th, 1970, in Prague, Czechoslovakia. Experienced investigators from Europe and America participated and 55 papers were presented. These contributions are divided into the following sections: the site of erythropoietin production, the regulation of erythropoietin production and utilization, the immunological properties of erythropoietin, the mechanism of action of erythropoietin, the kinetics of erythropoiesis, fetal erythropoiesis, the role of iron in haemoglobin synthesis, the initiation of haemoglobin synthesis, haem synthesis in erythroid cells, globin synthesis in erythroid cells.

The proceedings will be a valuable source of information and references for laboratory investigators and clinical haematologists.

H. R. Marti, *Aarau*

A. L. Copley and B. Blomback (ed.): **Thrombosis Research,** vol. 1, 1, February 1972.

This is the first issue of a new international journal on vascular obstruction, hemorrhage and hemostasis. The journal should allow a rapid publication of full lenght papers. A photographic process will accelerate publication and permit the use of four languages: English, French, German und Russian. Also the editorial responsibility will be divided among not less than 34 editors. Each editor has a personal responsibility in accepting a manuscript since his name will appear together with the acceptance date at the beginning of the paper.

In the first issue some aspects of the broad field covered by the new journal have been considered especially, however, factor VIII and related problems.

F. Duckert, *Basel*

J. M. Paulus (ed.): **Platelet Kinetics.** North Holland, Amsterdam 1971. 370 pp., 104 figs, 32 tables.

This is the second volume of the North Hollande Frontiers Series 'Clinical Studies'.

The volume (edited in collaboration with R. H. Aster and H. Paulus) is constituted by the Proceedings of Conference on platelet kinetics held in Liège, Belgium, on July 27–28, 1970. It must have been a very concentrated Conference since the volume is loaded with information dealing with all aspects of platelet kinetics. The proceedings are more than just clinical studies and concern to a large extent basic research. The different authors study carefully the platelet life span, age and destruction. The labelling of platelets and analytical mathematical methods for the estimation of platelet kinetics are presented with their advantages, disadvantages and pitfalls. Thrombopoiesis is discussed. The second part, particularly important, is reserved to the studies in humans of the megakaryocytes labels. From the more theo-

Index rerum ad Vol. 48

Bearbeitet von G. BOEHM, Basel

(B) = Book reviews – Buchbesprechungen – Livres nouveaux

lymphocyte receptors'. The first paper by DE WECK presents a hypothesis about the molecular events responsible for the stimulation of antigen sensitive cells in general. The next paper tries to analyse the T-cell receptors which are undefined until now but must exist. T-cells have specificity as shown in another paper by SCHLOSSMAN. Another subject are the allotypic antibodies. These antibodies which are directed against the cell-associated receptors for transplantation antigens are discussed by RAMSEIER and LINDENMANN. The last two papers deal with the events after recognition of antigen by T-cells: first, the in vitro reactions of T-cells (BRONDZ) and, second the products of T-cell activation, the mediators of cellular immunity (REMOLD).

The volume contains papers on topics which are highly active and controversial at the present time. All subjects are well treated. This stimulating collection can be highly recommend to every immunologist.

T. L. VISCHER, Geneva

Varia

Pappenheim-Preis

Die Deutsche Gesellschaft für Hämatologie hat den Pappenheim-Preis für 197[illegible] [illegible] Der Preis ist bestimmt für deutschsprachige Arbeiten [illegible] oder theoretischen Fragen der Hämatologie befassen und die neue, d.h. [illegible] Leistungen [illegible] 40 Jahre [illegible] Die Arbeiten sind anonym mit einem Kennwort unter Angabe der Geburts[illegible] [illegible] 1. März 1973 beim Schriftführer der Gesellschaft Dr. [illegible] [illegible] 12, D-1 Berlin 20 (BRD) eingereicht werden. Der Preis [illegible] 2000 DM (Acta haemat. 47: 256, 1972).

Index rerum ad Vol. 48

Bearbeitet von G. BOEHM, Basel

(B) = Book reviews – Buchbesprechungen – Livres nouveaux

Index autorum ad Vol. 48

(B) = Book reviews – Buchbesprechungen – Livres nouveaux